IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Robert MARTUZA *et al.*
Title:	REPLICATION-COMPETENT HERPES SIMPLEX VIRUS MEDIATES DESTRUCTION OF NEOPLASTIC CELLS
Appl. No.:	10/748,233
Filing Date:	12/31/2003
Examiner:	Wu Cheng Winston Shen
Art Unit:	1632
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DECLARATION UNDER 37 CFR § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Samuel D. Rabkin, hereby declare that:

1. I am a co-inventor of U.S. patent application serial No. 10/748,233 ("the application"), which, I understand, was filed on December 31, 2003, and benefits from a priority date of June 23, 1994.

2. I received my Bachelor of Science in Biology from University of Toronto (Toronto, Canada) in 1976. In 1978, I received my Master of Science in Microbiology from Hebrew University of Jerusalem (Jerusalem, Israel). Since then I have worked in the area of herpes simplex virus and its application for treating cancer. In 1983 I received my Ph.D. in Microbiology from University of Chicago (Chicago, IL). I am currently an associate professor of the Harvard Medical School and an associate virologist of Massachusetts General Hospital.

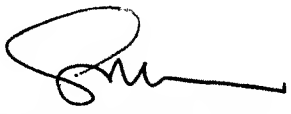
3. I understand that subject matter claimed in the application ("the claimed invention") is deemed by the U.S. Patent and Trademark Office to have been presaged by the contemporaneous literature and, hence, is unpatentable.

4. I have reference to evidence from the pre-filing literature, however, demonstrating that cytokines such as IL-1α, IL-2, IL-3, TNF, IFN-α, IFN-β, IFNγ, M-CSF-1 and GM-CSF were expected to have antiviral effects, such as protecting a host from herpes simplex virus (HSV) infection and preventing HSV replication in the host. In particular, Chatterjee *et al., J. Virol.* 56: 419-425 (1985), Exhibit A, show that IFN-α2 and IFN-β block HSV-1 replication. Ito *et al., Lymphokine Res.* 6: 309-318 (1987), abstract submitted herewith as Exhibit B, describe that tumor necrosis factor (TNF), alone or in combination with IFNγ, has antiviral activities against human herpes simplex virus (HSV) types 1 and 2, e.g., suppression of virus growth. Feduchi *et al., J. Virology* 63: 1354-59 (1989), Exhibit C, confirm that TNF and IFNγ exhibit synergistic inhibitory effects on HSV-1 replication. Chatterjee *et al., Virus Research* 12: 33-42 (1989), Exhibit D, report that human alpha interferon (IFN-α) A/D significantly reduces the replication and cell fusion induced by HSV-1. Kohl *et al., J. Infect. Dis.* 159: 239-47 (1989), abstract submitted as Exhibit E, show that interleukin-2 (IL-2) protects neonatal mice from HSV infection. Gangemi *et al., J. Interferon Res.* 9: 227-37 (1989), abstract submitted as Exhibit F, disclose that IFN-α B/D is highly effective in preventing viral replication and cell destruction induced by HSV-1 in human monocyte cultures. Iida *et al.*, *Vaccine* 7: 229-33 (1989), Exhibit G, demonstrate that GM-CSF has protective activity against herpes simplex viral infection in mice. Chan *et al., Immunology* 71: 358-63 (1990), Exhibit H, present that IL-3 markedly inhibits HSV-1 replication in primary mouse embryonic head cell cultures. Berkowitz *et al., Arch. Virol.* 124: 83-93 (1992), Exhibit I, conclude that IL-1α, IL-2, M-CSF-1, as well as combinations of IL-2 and M-CSF-1, are effective in protecting mice against HSV-1 infection.

5. In light of the above-mentioned evidence, it is my opinion, as an expert in the field of HSV-mediated therapies, that achieving an enhanced tumor therapy effect by combining an HSV with cytokines would have been unexpected, *circa* June of 1994. Thus, the claimed invention requires a cytokine-expressing HSV to infect and replicate in tumor cells, thereby to elicit an anti-tumor immune response, and yet the contemporaneous literature, which I

have summarized above, indicated that the cytokines would counter or decrease the prerequisite HSV infection and replication.

6. I declare that the statements made herein of my knowledge are true and all statements on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therein.



Samuel D. Rabkin

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Date

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Effect of Cloned Human Interferons on Protein Synthesis and Morphogenesis of Herpes Simplex Virus

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Pretreatment of human fibroblast cells with 100 U of either cloned human alpha-2 or beta interferon per ml for 24 h reduced the release of infectious herpes simplex virus type 1 by more than 99%. This inhibition in infectivity correlated well with the total number of extracellular virus particles released from treated cells as determined by DNA dot blot hybridization analysis. Electron microscopic observations of interferon-treated human fibroblast cells clearly demonstrated typical assembly of nucleocapsids inside the nucleus, even though very few mature extracellular particles were seen. Analysis of virus-specific proteins by the immunoblot technique showed that neither species of interferon had a significant inhibitory effect on the synthesis of major nucleocapsid proteins. However, the synthesis of specific glycoproteins (D and B) was drastically reduced or delayed in beta-interferon-treated cells. The results presented in this communication suggest that cloned human interferons block herpes simplex virus morphogenesis at a late stage and inhibit the release of particles from the treated cells.

Herpesvirus infections are among the most common of all human infections, causing a broad spectrum of diseases which range from asymptomatic to life threatening. With severe disease, herpes simplex encephalitis and neonatal herpes are associated with high mortality and morbidity. Of the five human herpesviruses, herpes simplex virus types 1 and 2 (HSV-1 and HSV-2, respectively) are particularly important because of frequent recurrences, with known transmission to susceptible individuals. With the recognition of these viruses as an important cause of human disease, numerous antiviral drugs have been tested as therapeutic agents and shown to be useful in treating some of these infections. However, viral resistance to drugs may occur, although it is of unknown biologic significance at present (6). We recently showed that cloned human alpha and beta interferons (IFN-α and IFN-β, respectively) block HSV-induced cell fusion and plaque formation in human fibroblast cells (5). Fish et al. (7) have also reported that human IFNs are effective against HSV infection, but the specific stage(s) of the virus life cycle which is affected by IFN was not addressed. Little is known about the effect of IFNs on DNA viruses in general and especially the molecular mechanisms by which IFN-treated cells prevent the replication of these viruses. Gloger and Panet (8) have recently reported that naturally produced human IFN-α inhibited the translation of HSV-specific proteins in treated cells. However, at the same time Munoz and Carrasco (15) reported that no major inhibition of HSV protein synthesis occurred in IFN-α-treated cells. In this communication, we demonstrate that both cloned human IFN-α and IFN-β significantly block the release of total extracellular particles from IFN-treated cells. Neither species of IFN had a significant inhibitory effect on the synthesis of major nucleocapsid proteins, but the synthesis of specific glycoproteins was drastically reduced or delayed. Electron microscopic observations of HSV-infected cells pretreated with these IFNs showed typical assembly of nucleocapsids within the nucleus. These results strongly suggest that these IFNs act in an unique manner in HSV-infected cells by blocking HSV-1 replication at a late stage in morphogenesis and preventing the release of virus particles from the treated cells.

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MATERIALS AND METHODS

Cell cultures and viruses. Human foreskin (HFS) cells were prepared by published procedures (18). African green monkey kidney (BS-C-1) cells were obtained from the American Type Culture Collection, Rockville, Md. HFS cells were grown in Eagle minimal essential medium containing 10% heat-inactivated fetal calf serum. BS-C-1 cells were grown in medium 199 also supplemented with 10% heat-inactivated fetal calf serum.

The F and MP strains of HSV-1 were kindly provided by B. Roizman, The University of Chicago, Chicago, Ill.

Reagents and radioisotopes. Reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories, Richmond, Calif. $Na^{125}I$ (15.5 mCi/μg of iodine) was purchased from Amersham Corp., Arlington Heights, Ill. [α-^{32}P]dCTP (800 Ci/mmol) and [α-^{32}P]dTTP (800 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. Cloned human IFN-α_2 (10^8 U/mg of protein) and IFN-β (10^9 U/mg of protein) were generously provided by J. Schwartz, Schering-Plough Corp., Bloomfield, N.J., and F. McCormick, Cetus Corp., Emeryville, Calif., respectively.

Antisera. Rabbit antiserum to HSV-1 and monospecific rabbit antisera to glycoproteins B and D (gB and gD, respectively) were kindly provided by B. Norrild, University of Copenhagen, Copenhagen, Denmark (23).

Electron microscopy. IFN-treated and untreated HFS cells grown in 60-mm-diameter dishes were processed for electron microscopy after infection with HSV-1. Cells were carefully washed with phosphate-buffered saline and fixed with 1% glutaraldehyde. Samples were postfixed with 1% osmium tetroxide and embedded in an epoxy resin mixture. Thin sections were then stained with uranyl acetate and lead citrate and were examined under a Philips EM 301 electron microscope.

Southern dot blot technique. Viral DNA samples treated

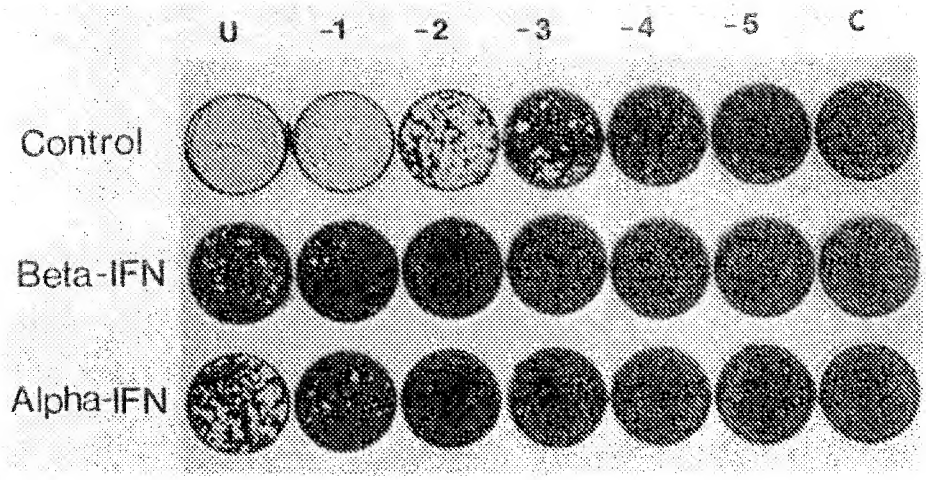


FIG. 1. Effect of cloned human IFNs on replication of HSV-1 in human cells. Human fibroblast cells were pretreated with either IFN-α_2 or IFN-β and then infected with the MP strain of HSV-1 as described in the text. Supernatants were collected, serially diluted, and then tested for their ability to form plaques in BS-C-1 cells. PFU per milliliter were calculated after the cells were stained with May-Grunwald-Giemsa stain as described by Neff and Enders (16).

with RNase A and ribonuclease T_1 were diluted and spotted onto nitrocellulose filter paper after they were denatured as described previously (2, 11, 21). Samples on nitrocellulose paper were then hybridized with ^{32}P-labeled nick-translated HSV-1 DNA (1×10^7 cpm/filter) for 18 h. Nick-translated probe was prepared by the standard procedure (14). The filter paper was washed thoroughly in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 42°C and then exposed to Kodak XR-2 film with a DuPont Cronex Hi-Plus intensifying screen.

Polyacrylamide gel electrophoresis and electrophoretic transfer of proteins to nitrocellulose filter paper (Western blotting). Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9.5%) as described by Chatterjee et al. (3). The fractionated proteins were then electrophoretically transferred (0.2 A for 5 h) to nitrocellulose paper. The procedure of Towbin et al. (22) was followed, with some modifications adapted from the method of Johnson et al. (10). After the transfer, nitrocellulose blots were blocked in Bovine Lacto Transfer Technique Optimizer (5% nonfat dry milk in phosphate-buffered saline with 0.001% sodium azide; BLOTTO) (10) and then reacted with selected antisera for 16 h at 37°C. Bound antibodies were detected by reacting the blots with ^{125}I-protein A for 1 h at 37°C followed by autoradiography as described above. Protein A iodination was carried out by the procedure described by Greenwood et al. (9).

RESULTS

Effect of cloned human IFNs on the replication of HSV-1 in human cells. To quantitatively determine the effect of cloned IFNs on the replication of HSV-1, HFS cells were pretreated with 100 U of either cloned IFN-α_2 or IFN-β per ml for 18 h. One set of cells served as an untreated control. All cells were then infected with the MP strain of HSV-1 (multiplicity of infection, approximately 2). Supernatant harvests were collected 24 h postinfection, and the quantities of infectious virus released from IFN-treated and untreated cells were determined by plaque assay on BS-C-1 cells. The result of such an experiment is shown in Fig. 1. Both IFN-α_2 and IFN-β inhibited the release of infectious HSV-1 particles from HFS cells more than 500-fold.

Cloned human IFNs block the release of total extracellular particles. Since the production of infectious virus was inhibited more than 500-fold by cloned human IFNs, it was necessary to determine whether any extracellular noninfectious particles were formed in the presence of these IFNs. HFS cells were pretreated with 100 U of either IFN-α_2 or IFN-β per ml for 18 h and then infected with the MP strain of HSV-1 (multiplicity of infection, approximately 2). One set of cells was kept as an uninfected control. Supernatant harvests were collected 24 h postinfection and clarified, and the virus was then pelleted by centrifugation at 45,000 rpm for 1 h. Viral DNA was extracted from the IFN-treated and untreated samples, treated with RNase A and ribonuclease T_1, and then processed for the DNA dot blot hybridization assay as described in Materials and Methods. The result of this experiment showed a greater than 90% reduction (calculated from ^{32}P counts of each spot) in the release of total extracellular virus particles (Fig. 2). Identical results were observed when extracellular viral proteins were assayed for by immunoblot (data not shown). Thus the block in replication appears to occur before the release of virus from treated cells.

Effect of cloned IFNs on the assembly of nucleocapsids. Since with several retroviruses IFN blocks the release of assembled virus particles from the plasma membrane (20; S. Chatterjee and E. Hunter, Meet. RNA Tumor Viruses, Cold Spring Harbor Laboratory abstr. no. 110, 1983), we were interested in determining whether a similar phenomenon occurred in HSV-infected cells. HFS cells were treated with 100 U of either cloned IFN-α_2 or IFN-β per ml and then infected with the MP strain of HSV-1 as in the previous experiment. The cells were processed for electron microscopy at 24 h postinfection as described in Materials and Methods. The electron micrographs showed the presence of assembled HSV cores inside the nuclei of both the IFN-treated and untreated cells (Fig. 3). However, while in the untreated cells numerous extracellular particles (Fig. 3a) could be observed, only a very few extracellular viral particles in IFN-α-treated cells and only intranuclear cores in IFN-β-treated cells were seen (Fig. 3b and c). These observations correlate well with the results of the DNA dot blot hybridization experiments described previously (5) (Fig.

2) and support the hypothesis that both of these IFNs block HSV-1 replication at a late stage in morphogenesis and in some way inhibit the exit of viral cores from the nucleus of treated cells.

Synthesis of virus-specific proteins in IFN-treated human cells. To determine whether the block observed in virus replication could be correlated with a reduction in the synthesis of specific viral coded proteins and thus prevent normal virus assembly and transport, immunoblotting experiments were performed as described below. Monolayers of HFS cells were treated with 100 U of either IFN-α_2 or IFN-β per ml for 18 h and then infected with the MP strain of HSV-1 as before. One set of cells served as an uninfected control. Cell lysates collected at 18 and 48 h postinfection were processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent immunoblotting as described in Materials and Methods. The blots from this experiment were reacted with rabbit anti-HSV-1 antiserum and ^{125}I-protein A sequentially, and the resulting autoradiograms are displayed in Fig. 4a. Although delay in the appearance of some viral proteins was observed at 18 h postinfection in IFN-β-treated cells, essentially all the major capsid proteins of HSV were synthesized by 48 h postinfection in this set. Indeed, in other experiments, in which the F strain of HSV-1 was used, less significant differences were seen between untreated and IFN-β-treated samples (Fig. 4b). Thus, while these results show that IFN-β slightly delayed the synthesis of HSV-specific proteins in human fibroblast cells, it is clear that by 48 h a majority of the capsid proteins were being synthesized normally. Since it was possible that the extended incubation time (48 h) after HSV infection had increased the level of replication of HSV in the IFN-treated cells, parallel plaque assays were carried out to quantitate the amount of infectious virus released in this experiment. The supernatants collected at 18 and 48 h postinfection from the plates (before lysis of the cells) were tested for their ability to form plaques in BS-C-1 cells. A significant reduction (greater than 100-fold for IFN-β) in the release of infectious virus from the IFN-treated cells was observed at 18, 48, and even 72 h postinfection (Table 1). These results demonstrate that in IFN-treated cells, at times when infectious virus release from cells is reduced more than 100-fold, the major capsid polypeptides of HSV are being synthesized normally, which is consistent with the electron microscopic observation of assembled virus cores in such treated cells.

Synthesis of virus-specific glycoproteins in IFN-treated human cells. Two possibilities could be envisioned to explain the lack of core envelopment and transport in IFN-treated cells. First, IFN treatment could cause a reduction in the fluidity of the nuclear membrane and thus prevent normal HSV morphogenesis. We have shown previously that IFN-α and IFN-β reduce the fluidity of cellular membranes, and while this has been most definitively demonstrated at the plasma membrane (4, 19, 24), similar changes may also occur at other membranes. Second, viral glycoprotein insertion into the inner nuclear membrane may be essential for core recognition and envelopment in a similar manner to that postulated for the vesicular stomatitis virus G protein. A block in the synthesis or migration or both of such proteins would thus interfere with virus assembly. Since the immunoblotting experiments with rabbit anti-HSV-1 antiserum specifically provided no information on virus glycoprotein expression, we next analyzed the same cell lysates for the status of gB and gD by using monospecific antiserum against these proteins. The IFN-β-treated cell lysates (18 and 48 h postinfection) prepared earlier were subjected to polyacrylamide gel electrophoresis, and the fractionated proteins were transferred to nitrocellulose paper. Two sets of blots were prepared; one set was reacted with rabbit anti-gB antiserum, whereas the other was reacted with rabbit anti-gD antiserum. The results of this experiment show that unlike the levels of the capsid proteins, the levels of gD in the lysates at both periods were significantly reduced by IFN-β (Fig. 5a). The amount of gB was also significantly inhibited at 18 h postinfecion, but after further incubation (to 48 h) the levels of this protein rose to those of untreated cells (Fig. 5b). More importantly, both the pre-gB and the mature gB could be seen in the lysates, indicating that intracellular transport of gB (at least to the Golgi complex) was occurring normally. Similar experiments with IFN-α also demonstrated a reduction in the amount of gB and gD in the lysates, although not as significant as that observed after IFN-β treatment.

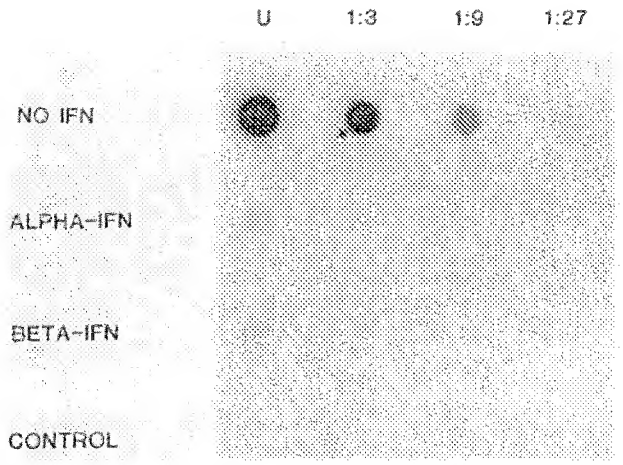
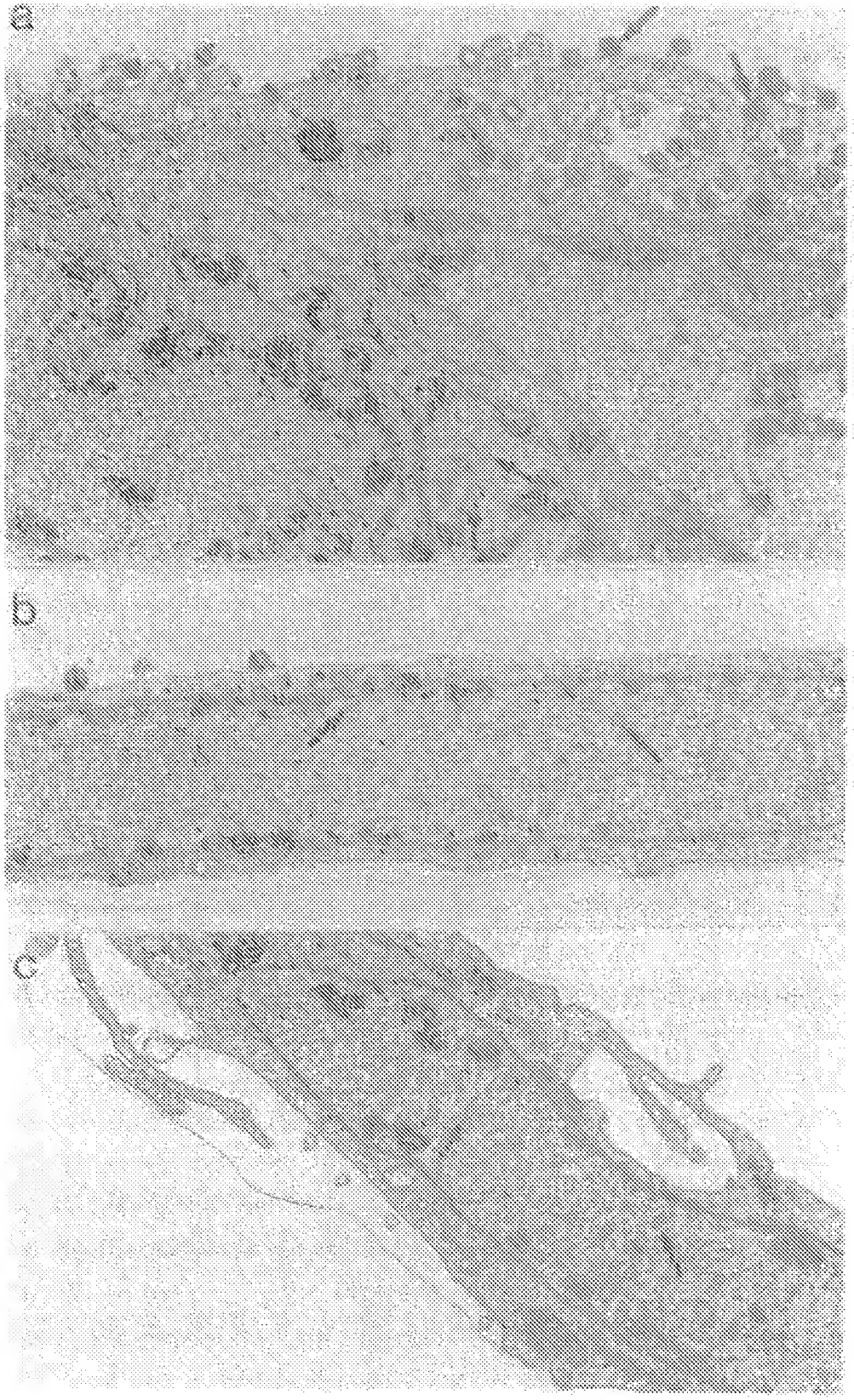


FIG. 2. Effect of cloned human IFNs on the release of extracellular virus particles. Human fibroblast cells pretreated with IFN-α_2 or IFN-β were infected with the MP strain of HSV-1 as described in the text. One set of cells served as an uninfected control. Supernatant fluids from each set were collected and clarified, and any virus present was pelleted by centrifugation. Viral DNA was extracted, serially diluted, and then processed for DNA dot blot hybridization as described in the text.

DISCUSSION

Little is known about the exact molecular mechanisms by which IFN prevents DNA virus replication. The results described in this communication, however, clearly demonstrate the stage(s) at which HSV replication is blocked by IFN. We have shown that both cloned IFN-α_2 and IFN-β significantly inhibit the replication of HSV-1 in human cells. Similar results (with IFN-α) have also been reported by Fish et al. (7), but the specific stage at which replication was affected by IFN in those experiments was not described. We show here that the inhibition of virus replication involves a block at a late stage in HSV morphogenesis, specifically at the level of virus core exit from the nucleus of IFN-treated cells. This conclusion is based on the fact that no extracellular particles were released from the IFN-treated cells, and electron microscopy demonstrated the assembly of nucleocapsids inside the nucleus of the IFN-treated cells. Consistent with these observations, IFN-treated cells showed no major differences in the nucleocapsid protein profile compared with that of untreated cells at times when the replica-



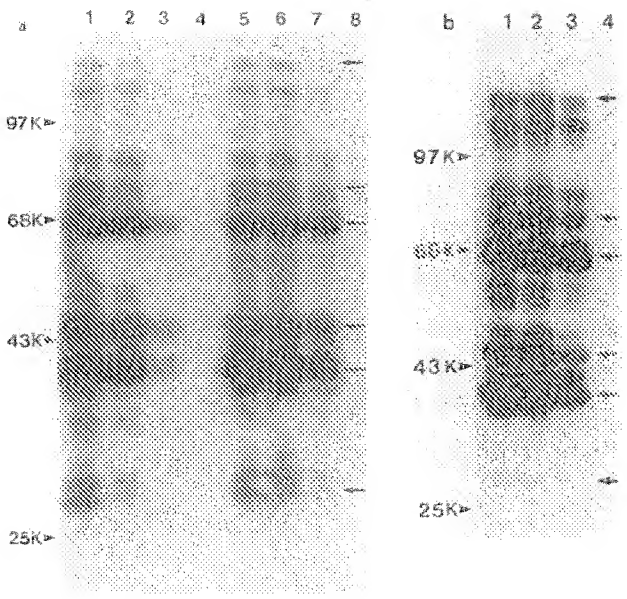


FIG. 4. Effect of cloned human IFNs on the synthesis of HSV-1 specific proteins in IFN-treated and -untreated human cells. (a) Human fibroblast cells were pretreated with 100 U of either IFN-α_2 or IFN-β per ml and then infected with the MP strain as described in the text. One set of cells served as an uninfected control. Cell lysates collected 18 and 48 h postinfection were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently processed for immunoblotting. The blot was reacted with rabbit anti-HSV-1 antiserum. Lanes: 1 through 4, 18 h postinfection; 5 through 8, 48 h postinfection; 1 and 5, no IFN; 2 and 6, INF-α; 3 and 7, IFN-β; 4 and 8, uninfected control. The major capsid proteins are indicated by arrows. (b) The experimental procedure was essentially same as described above, except that strain F of HSV-1 was used instead of strain MP and the cell lysates were collected at 24 h postinfection. Lanes: 1, no IFN; 2, IFN-α; 3, IFN-β; 4, control. The major capsid proteins are indicated by arrows.

tion of infectious virus was still inhibited more than 100-fold. The presence of virus-specific proteins in the IFN-treated cells also ruled out the possibility that the block was at the stage of virus penetration or at an early stage before viral DNA synthesis. This is in agreement with our previous finding, when we reported that similar levels of viral DNA were synthesized in IFN-treated and untreated cells. Since it has been postulated that viral glycoproteins might act as nucleation points for virus budding and envelopment in several enveloped virus systems, we investigated, through the use of monospecific antisera to two HSV glycoproteins, whether any specific effects on glycoprotein synthesis could be observed. Surprisingly, both gB and gD are present in significantly reduced amounts at 18 h postinfection in IFN-β-treated cells infected with HSV-1, and while the levels of gB appear to recover in the next 30 h, those of gD still remain significantly reduced. This effect on viral glycoprotein biosynthesis could also explain the drastic reduction in cell-to-

TABLE 1. Effect of cloned human IFN-α_2 and IFN-β on replication of HSV-1 as a function of time[a]

Incubation time (hr)	Addition	% Inhibition
18	None	0
	IFN-β (100 U/ml)	>99
	IFN-α_2 (100 U/ml)	>99
48	None	0
	IFN-β (100 U/ml)	>99
	IFN-α_2 (100 U/ml)	>94
72	None	0
	IFN-β (100 U/ml)	>99
	IFN-α_2 (100 U/ml)	>87

[a] Human cells were pretreated with different IFNs and infected with the MP strain of HSV-1 as described in the text. Supernatant fluids were collected 18, 48, and 72 h postinfection and tested for their ability to form plaques in BS-C-1 cells.

cell fusion observed in IFN-treated cells (5), since Noble et al. (17) have recently shown, through the use of monoclonal antibodies to gB and gD, that gD is the HSV-1 protein responsible for cell fusion activity. An effect of IFN on the viral glycoproteins thus could have a pleotropic action on both viral assembly-morphogenesis and cell fusion. At present, it is not possible to distinguish between a reduction in the rate of synthesis of gD or an increased rate of turnover of gD, since immunoblotting provides only a measure of steady-state levels of the protein. Pulse chase immunoprecipitation studies and an analysis of gD mRNA levels should provide information on the molecular mechanisms involved in the cause of its reduced levels.

The mechanism of IFN action on HSV replication is clearly quite different from that in other viral systems. The well-documented stimulation of a 2,5 oligoadenylate-activated endonuclease by IFNs and a protein kinase that appears to be effective in blocking translation of certain RNA viral proteins (1, 12) does not appear to be important in this case, since high levels of viral protein synthesis are observed in IFN-treated cells. Moreover, the block appears to be different from that observed in certain retroviruses, in which virus particles are assembled and enveloped in the presence of IFN but are inhibited at the stage of virus release from the cell (20; Chatterjee and Hunter, Meet, RNA Tumor Viruses 1983). We have not observed HSV cores partially enveloped by the nuclear membrane in IFN-treated cells; they appear instead to be scattered randomly through the nucleus. An effect of IFN on viral glycoprotein biosynthesis has been reported by Maheshwari and Friedman (13), who demonstrated that in mouse L cells treated with low levels of IFN, the vesicular stomatitis virus G protein was not glycosylated, but was synthesized and incorporated into virus particles, an event not observed in our studies with HSV-1. It is possible that IFN affects a unique step in the complicated transcription-translation cascade that regulates HSV-1 gene expression and in this way prevents normal expression of gB and gD. Two papers reporting conflicting

FIG. 3. Electron microscopic observations of virus particles present in thin sections of IFN-treated and -untreated human cells infected with HSV-1. (a) Thin section of HFS cells infected with the MP strain without IFN treatment, showing mature HSV particles (arrows). (b) and (c) Thin sections of HFS cells pretreated with either cloned IFN-α_2 or IFN-β, respectively, and then infected with the same strain. Note the distinct HSV cores assembled in nucleus (arrows). Magnification, ×28,000.

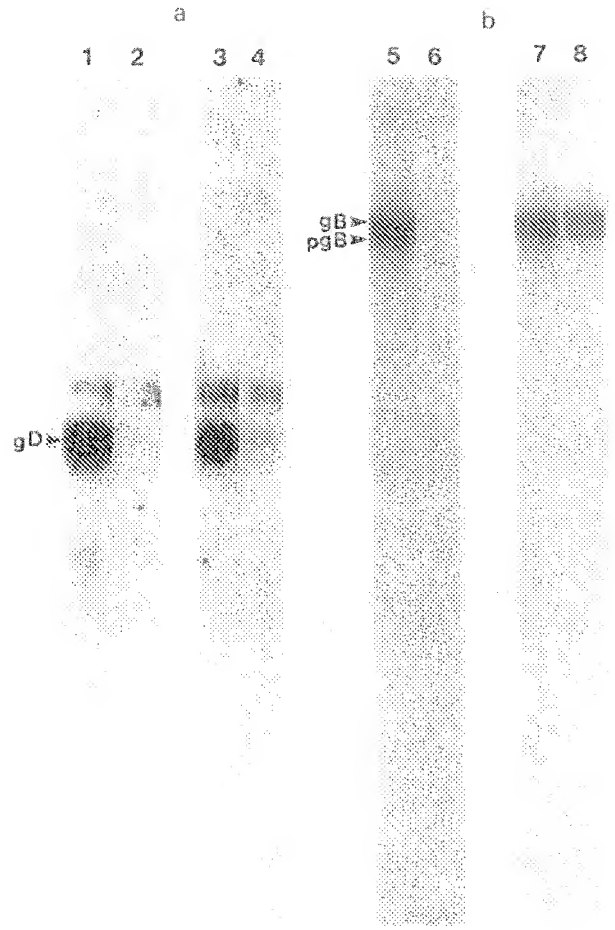


FIG. 5. Effect of cloned human IFN-β on the synthesis of gB and gD in IFN-treated and -untreated human cells. The IFN-β-treated cell lysates used in the experiment described in Fig. 4a were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in duplicate and subsequently processed for immunoblotting. The blots were reacted with either rabbit anti-gD (a) or rabbit anti-gB antiserum (b). Lanes: 1, 2, 5, and 6, 18 h postinfection; 3, 4, 7, and 8, 48 h postinfection; 1, 3, 5, and 7, no IFN; 2, 4, 6, and 8, IFN-β.

results have recently been published on the effects of IFN on HSV-1 replication. Gloger and Panet (8) observed an inhibition of immediate-early (α) polypeptide biosynthesis and a subsequent reduction of early (β) and late (γ) gene products. While those authors used significantly higher levels of IFN (2,000 U/ml), they analyzed polypeptide biosynthesis at 5 h postinfection and may have observed the delay in polypeptide biosynthesis we observed with IFN-β. However, the concentration of IFN used in our studies had no significant effect on cellular viability. Munoz and Carrasco (15), on the other hand, report no effect of natural human IFN-α on HSV-1 polypeptide synthesis or virus assembly, but rather the release of normal levels of noninfectious virus particles. We estimate from DNA dot blot experiment that less than 5% of the normal levels of virus particles were released in our experiments. While it is not possible to reconcile the results of the two studies at present, it should be noted that we have consistently observed a 10-fold-greater inhibition of infectious virus when using 4-fold less IFN than reported by Munoz and Carrasco (15). The differences in our results may thus reflect the differences in the IFN preparations. While the experiments described here clearly show that the level of expression of HSV-1 glycoproteins is significantly reduced in IFN-treated cells, we cannot at present conclude that this represents the block in viral assembly. The level of other HSV-1 gene products not quantifiable in the current experiments may also be affected in IFN-treated cells, and it could be these gene products that are required for normal nucleocapsid assembly and transport. Alternatively, membrane fluidity changes, which clearly occur in IFN-treated cells (4, 19, 24), could play a role in the disruption of HSV morphogenesis. Experiments aimed at addressing these possibilities are at present under way.

ACKNOWLEDGMENTS

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Links

Antiviral effects of recombinant human tumor necrosis factor.

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The antiviral action of recombinant human tumor necrosis factor (TNF) was studied using assay systems to determine inhibition of viral cytopathic effect (CPE), as well as suppression of virus growth measured by plaque assays. TNF was cloned and prepared by Asahi Chemical Industry, Japan. Antiviral activity against human herpes simplex virus (HSV) types 1 and 2, cytomegalovirus (CMV), varicella-zoster virus (VZ), vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMC), was demonstrated in human diploid fibroblasts following pretreatment with TNF overnight. The antiviral action was completely neutralized by anti-interferon (IFN)-beta serum, but not by anti-IFN-alpha or -gamma antibodies. This suggested the induction of IFN-beta by TNF. The antiviral action was synergistically enhanced by human IFN-gamma. Several non-human cell lines were tested but 10 of 11 failed to be protected from VSV- and/or EMC-induced CPE following pretreatment by TNF. The anticellular effects of TNF were tested in human and in non-human tumor cell lines. The results indicate that the susceptibility of cells to the two activities of TNF, antiviral and anticellular, was distinct, and that antiviral activity of TNF is more species-specific than its anticellular action.

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EXHIBIT C

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Human Gamma Interferon and Tumor Necrosis Factor Exert a Synergistic Blockade on the Replication of Herpes Simplex Virus

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The replication of herpes simplex virus type 1 (HSV-1) is not inhibited in either HeLa or HEp-2 cells treated with human alpha interferon (HuIFN-α), particularly when high multiplicities of infection are used. However, HuIFN-γ partially inhibits HSV-1 translation in HEp-2 cells infected at low multiplicities. Under these conditions, the transcription of genes α22, TK, and γ0 is greatly diminished. The combined addition of human tumor necrosis factor (TNF) and HuIFN-γ to HEp-2 cells exerts a synergistic inhibition of HSV-1 translation. Cells treated with both cytokines continue synthesizing cellular proteins, even 20 h after HSV-1 infection. As little as 10 U of IFN-γ per ml blocked HSV-1 DNA replication, provided that TNF was also present in the medium. Analyses of HSV-1 gene transcription suggest that the action of both TNF and IFN-γ blocked a step that comes at or prior to early HSV-1 gene expression. This early step in HSV-1 replication inhibited by TNF and IFN-γ occurs after virus attachment and entry into cells, since the internalization of radioactive HSV-1 virion particles was not blocked by the presence of the two cytokines. Therefore, we conclude that the synergistic action of TNF plus IFN-γ affects a step in HSV-1 replication that comes after virus entry but before or at the transcription of immediate-early genes.

The response of animal viruses to the inhibitory effects of interferon (IFN) greatly depends on a number of variables. The IFN species used to block virus replication is an important factor, since some viruses, adenoviruses for example, are inhibited by IFN-γ, but not by IFN-α (14). The virus-cell system is another factor of interest, because not all animal viruses studied are equally sensitive to IFN (11, 19). In general, viruses containing RNA molecules as the genome are more prone to be inhibited by IFN than DNA-containing viruses (11, 19). The replication of vesicular stomatitis virus or poliovirus is very sensitive to all IFN species assayed in the different cell types used (11, 19). On the other hand, the translation of reovirus type 3 is not inhibited by human alpha interferon (HuIFN-α) in HeLa cells, but it is blocked by murine alpha-beta interferon (MuIFN-α-β) in mouse cells (9, 18). The resistance of reovirus replication to IFN is not due to reversion of the antiviral state (9). This behavior contrasts with that of most DNA-containing animal viruses studied (8, 22). Vaccinia virus and adenoviruses owe their resistance to IFN to the fact that they are able to revert the antiviral state established in cells treated with IFN (8, 22). For herpesviruses the situation is more complex, and the response to IFN largely depends on the system under study. Herpes simplex virus type 1 (HSV-1) replicates well in HeLa cells treated with HuIFN-α; neither DNA replication nor viral protein synthesis is inhibited in these cells (4, 17), but the virions made in cells treated with IFN-α are unable to replicate (17). Chatterjee et al. (4) found a reduction in the amount of glycoproteins D and B of HSV-1 and a drastic inhibition of the release of mature extracellular particles. More recently, it was found that the transcription of HSV-1 early genes was inhibited in HeLa cells treated with very high doses (4,000 U/ml) of HuIFN-αA and infected at low multiplicity (20). The use of another cell line, Chang cells, shows that high doses (100 to 1,000 U/ml) of HuIFN-α or HuIFN-β do block the growth of HSV-1. Consequently, no viral proteins appear late in infection when Chang cells are treated with 300 or 1,000 U of HuIFN-β per ml (3). HSV-1 replication is sensitive to IFN in mouse and human macrophages (5, 6, 15, 25). Thus, primary cultures of splenic mouse macrophages treated with MuIFN-α-β do not express immediate-early genes of HSV-1 (15). Treatment of macrophages with IFN-γ in combination with IFN-α or IFN-β resulted in a synergistic inhibition of HSV-1 growth (5). Therefore, the behavior of HSV-1 in human or mouse cells treated with a variety of IFN preparations is different.

The search for different cytokine combinations which are more effective in blocking HSV-1 replication in human cells may lead to an improvement of the available chemotherapy against this important human pathogen. In this regard, a new cytokine known as tumor necrosis factor (TNF) has shown some promise, used either alone or in combination with different IFNs against a number of animal viruses (13, 30). TNF is a cytokine with pleiotropic effects (1, 21). TNF is a 17,000-dalton polypeptide synthesized by macrophages in response to several agents such as lipopolysaccharide or tubercle bacilli (1, 21). This protein is best known for its antitumor effects in vivo and for its in vitro cytotoxicity to a number of transformed cell lines (1, 21). In addition, TNF also stimulates the growth of normal fibroblasts (26, 28), inhibits the synthesis of lipoprotein lipase (1), and activates polymorphonuclear leukocytes (23). Recently, an antiviral activity of TNF was described (13, 30). TNF alone shows an antiviral effect in several cell lines against a number of animal viruses, such as vesicular stomatitis virus, encephalomyocarditis virus, adenovirus type 2, or HSV-2 (13, 30). More interestingly, the antiviral effects of TNF are enhanced synergistically by IFN-γ (30). These results prompted an investigation to clarify the action of TNF and IFN-γ against HSV-1 in human fibroblasts, and the results are presented here.

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MATERIALS AND METHODS

Cells and viruses. HEp-2 and Vero cells were grown in petri dishes (Nunc, Roskilde, Denmark) containing 10 ml of Dulbecco modified Eagle medium (E4D) supplemented with 10% calf serum (GIBCO Laboratories, Grand Island, N.Y.) and incubated at 37°C in a 5% CO_2 atmosphere.

HSV-1 was grown on Vero cells in E4D medium supplemented with 2% calf serum (E4D2). The fraction obtained after removal of cell debris by low-speed centrifugation was used as the source of virus. Virus preparations were titrated by plaque assay.

Plasmids. The 2-kilobase (kb) *Pst*I (α22 gene), 2.4-kb *Eco*RI (TK gene), and 3-kb *Hin*dIII (γ0 gene) restriction fragments obtained from HSV-1 genome digestion and cloned in pBR322, pACYC184, and pACYC177, respectively, were generously provided by E. Tabarés (Madrid, Spain).

IFNs and TNF. Human lymphoblastoid interferon, HuIFN-(Ly)-α (1.7×10^6 U/ml), was a generous gift from N. Finter (Wellcome Research Laboratories, Beckenham, England). Recombinant human gamma interferon, rHuIFN-γ (2×10^7 U/ml), and recombinant human tumor necrosis factor, rHuTNF-α (6×10^7 U/mg), were generously provided by G. R. Adolf (Ernst-Boehringer-Institut für Arzneimittel-Forschung). Human fibroblast interferon, HuIFN-β (5×10^4 U/ml), was from Lee Biomolecular Research Laboratories (San Diego, Calif.).

Conditions of infection. HEp-2 cells, grown in 96-well, 60- or 100-mm-diameter dishes, were infected with HSV-1 at the multiplicity of infection (MOI) indicated in each experiment in the Results section. After 1 h of incubation at 37°C, the medium was removed, and E4D2 was added. The time of virus addition was considered −1 h, and zero time was taken to be the time when the virus was removed. IFN and TNF treatment was done 20 to 24 h before infection at the concentration described in the figure legends, in E4D2. Medium with IFN and TNF was removed, and the infection was done as described above.

Analysis of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. HEp-2 cells grown in 96-well dishes were incubated with 50 μl of methionine-free medium and 1.25 μCi of [^{35}S]methionine (1.45 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) for 1 or 2 h at the time after infection indicated in each experiment. Cell monolayers were dissolved in 50 μl of sample buffer (62.5 mM Tris [pH 6.8], 2% sodium dodecyl sulfate, 0.1 M dithiothreitol, 17% glycerol, 0.024% bromophenol blue). Samples were heated at 90°C for 5 min, and 10 μl was applied to a 15% polyacrylamide gel and run overnight at 100 V/20 cm. Fluorography was carried out in 20% (wt/vol) 2,5-diphenyloxazole in dimethyl sulfoxide. The gels were finally dried and exposed with Kodak films (10).

RNA isolation and analysis. HEp-2 cells were grown in 100-mm dishes and infected at 5 PFU per cell. At the times indicated, cells were lysed in a buffer containing 10 mM Tris hydrochloride (pH 7.8), 1 mM EDTA, 150 mM NaCl, and 0.65% Nonidet P-40. After nuclei were removed by centrifugation at $2,000 \times g$ for 5 min, supernatants were mixed with an equal volume of buffer containing 20 mM Tris hydrochloride (pH 7.8), 350 mM NaCl, 20 mM EDTA, and 1% sodium dodecyl sulfate. Samples were extracted three times with a mixture of phenol and chloroform (24:1, vol/vol), and the RNA was precipitated with ethanol (7, 12). Dot blot analysis with ^{32}P-labeled nick-translated HSV-1 TK DNA, α22 DNA, and γ0 DNA was performed as previously described (27).

DNA isolation and analysis. HEp-2 cells were grown in 60-mm dishes and infected with HSV-1 at 30 PFU/ml. At 16 h postinfection (p.i.), cells were collected, and DNA was isolated essentially as described previously (2). DNA was immobilized onto nitrocellulose membranes with a microfiltration apparatus (Bio-Rad Laboratories, Richmond, Calif.) and subjected to hybridization to nick-translated TK DNA.

Preparation of radiolabeled viral particles. HEp-2 cells grown in 100-mm dishes and infected with 10 PFU of HSV-1 per cell were labeled with 50 μCi of [^{35}S]methionine per ml from 10 to 24 h p.i. Cells were scraped, and the fraction obtained after removal of cell debris was centrifuged over a 30% dextran cushion at 40,000 rpm for 3 h at 4°C in a 65 Ti rotor (Beckman Instruments, Inc., Fullerton, Calif.). The pellet was suspended in Dulbecco modified Eagle medium and used as radiolabeled virions to measure virus entry.

Virus entry. The entry of virus into cells was measured by estimating the trichloroacetic acid-precipitable radioactivity in the cell monolayer at different times after infection with radiolabeled virus (see above) at 37°C. The values obtained at 4°C were taken as a measure of virus adsorption. At the indicated times, the medium was removed and cell monolayers on 24-well dishes were washed with phosphate-buffered saline–Dulbecco modified Eagle medium and 0.0025% trypsin–0.08% EDTA in Dulbecco modified Eagle medium, precipitated with 5% trichloroacetic acid, washed with ethanol, solubilized with 0.1% NaOH–1% sodium dodecyl sulfate, and counted in an Intertechnique scintillation spectrometer.

RESULTS

Effects of different IFNs on HSV-1 replication. Previous experiments indicated that HSV-1 was able to replicate and synthesize proteins in human fibroblasts (HeLa cells) treated with IFN-α (4, 17). To analyze the sensitivity of HSV-1 replication to HuIFN-α, HuIFN-β, and HuIFN-γ, HEp-2 cells were treated with a high concentration (400 U/ml) of different human IFNs and were infected with HSV-1 at several multiplicities. As found in previous studies, the synthesis of HSV-1 proteins is very resistant to treatment with HuIFN-α (Fig. 1). This phenomenon is dependent upon the MOI used. When cells are infected at low MOI, the expression of some viral proteins is blocked. The increase in MOI overcomes the inhibition of HSV-1 protein synthesis in cells treated with IFN-α. It is surprising to find that IFN-β behaves as IFN-γ and that both IFNs show a higher activity against HSV-1 than does IFN-α. At low MOI most of the HSV-1 proteins are not evident when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, whereas at high MOI, viral protein synthesis is similar to that of control cells not treated with IFN. These results reinforce the idea that HSV-1 replication is quite resistant to IFN, particularly to IFN-α, as far as translation is concerned.

To compare the effect of IFN-α and IFN-γ on the transcription of HSV-1 genes, the experiment shown in Fig. 2B was conducted. Total RNA from infected cells treated or not treated with IFN was extracted at 4 and 8 h p.i. and hybridized with probes derived from α22, TK, and γ0 HSV-1 genes (29). Inhibitors of protein synthesis such as cycloheximide block the expression of β and γ genes but not the transcription of the α class (Fig. 2A). On the other hand, inhibitors of viral DNA replication such as phosphonoformate have an inhibitory effect on γ gene expression, but they do not block the transcription of α and β genes. HSV-1 expresses the γ0 and TK genes at control levels in HEp-2

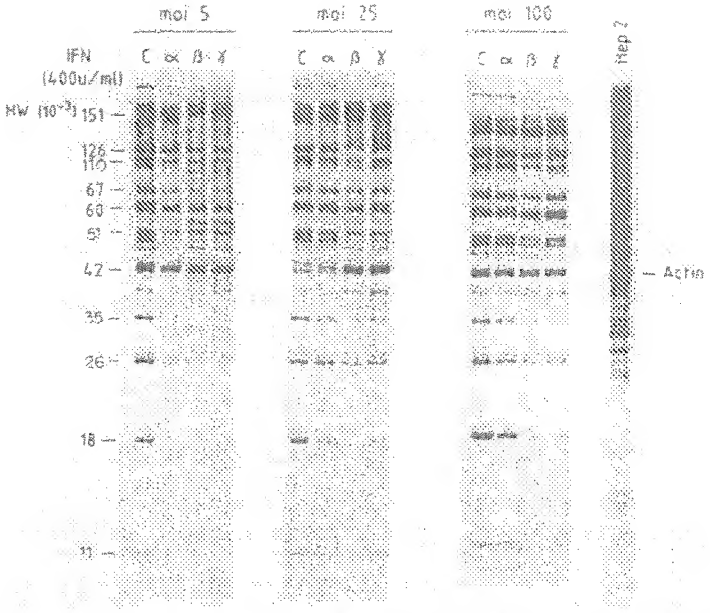

FIG. 1. Effect of IFNs on protein synthesis in HEp-2 cells infected with HSV-1. Cells grown in 96-well dishes were treated with IFNs 20 h before infection at the MOI indicated. Metabolic labeling was carried out for 2 h at 15 h p.i. with [^{35}S]methionine, and proteins were analyzed as described in Materials and Methods. C, Control without IFN. The numbers indicate molecular size in kilodaltons.

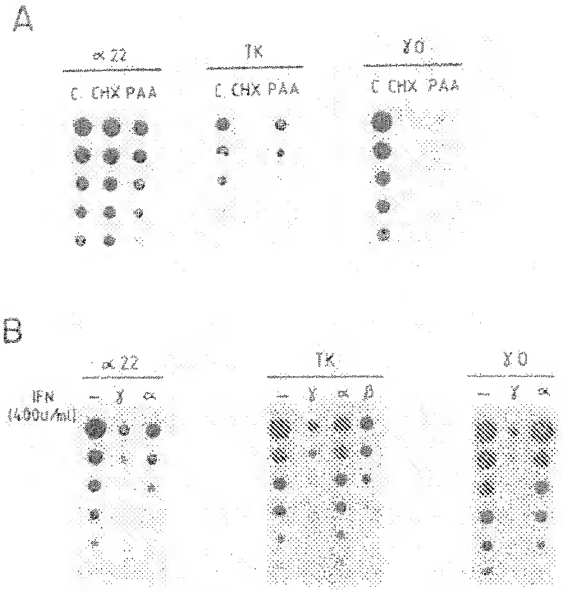

FIG. 2. Effects of different IFNs on the transcription of different HSV-1 genes. Cells were infected with 5 PFU of HSV-1 per cell, and mRNA was extracted at 4 h p.i. for the α22 gene blots and at 8 h p.i. for the TK and γ0 gene blots. (A) Effect of cycloheximide (CHX) (5×10^{-5} M) and phosphonoacetic acid (PAA) (250 μg/ml) on transcription of HSV-1 immediately-early (α22), early (TK), and late (γ0) genes. (B) Effect of IFNs on transcription of HSV-1 genes. Cells grown in 100-mm dishes were treated with IFN 24 h before infection. C, Control; –, control without treatment.

cells treated with 400 U of HuIFN-α per ml and infected at low MOI of HSV-1 (Fig. 2B). The synthesis of the α22 gene, however, is partially inhibited by HuIFN-α, suggesting that under these conditions IFN-α also has some inhibitory effect on the expression of early genes. The sensitivity of HSV-1 gene expression to IFN-γ is clearly apparent when the transcription of the three genes is analyzed (Fig. 2). An inhibition of more than eight times in the expression of α22 was observed, and consequently, the expression of the TK and γ0 genes was also blocked. These results suggest that the replication of HSV-1 in human fibroblasts can be partially inhibited by IFN-γ when a low MOI is used. Therefore, HSV-1 in this system somehow resembles the situation in mouse macrophages treated with MuIFN-β. These experiments clearly reinforce the idea that the final outcome of HSV-1 replication in a given cell type depends not only on the species of IFN used but also on other variables, such as the concentration of IFN, the MOI of the experiment, and so on (3, 11, 14, 19).

Synergistic effect of TNF and IFN-γ against HSV-1 replication. The combined addition of human TNF and HuIFN-γ leads to a synergistic inhibition of HSV-1 replication (1, 30). Treatment of HEp-2 cells with TNF alone, even at concentrations of 100 ng/ml, had no effect on HSV-1 protein synthesis (Fig. 3). In agreement with the results shown in Fig. 1 and 2, neither IFN-α nor IFN-γ at low concentrations (10 U/ml) prevented the appearance of HSV-1 late proteins. Strikingly, the combination of TNF and IFN-γ fully suppressed the synthesis of HSV-1 proteins (Fig. 3), indicating a synergistic action. This effect was not observed when IFN-α was added, further suggesting that the activities of IFN-α and IFN-γ against DNA-containing viruses are not similar. Since the MOI used was an important factor determining the resistance or sensitivity of HSV-1 late-protein synthesis to IFN-γ, we analyzed the effect of this factor on HSV-1 translation (Fig. 4). Again, HSV-1 proteins were

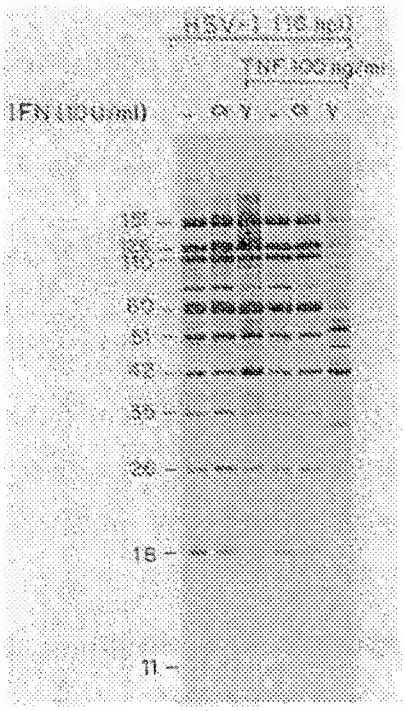

FIG. 3. Effect of IFN-α, IFN-γ, and TNF treatment on protein synthesis in HEp-2 cells infected with HSV-1. Cells were grown in 96-well dishes and treated with TNF (100 ng/ml), IFN-α (10 U/ml), and IFN-γ (10 U/ml) 24 h before infection with 25 PFU per cell. Cells were labeled at 16 to 18 h p.i. with [^{35}P]methionine, and proteins were analyzed as described in Materials and Methods. −, Control without teatment. The numbers indicate molecular size in kilodaltons.

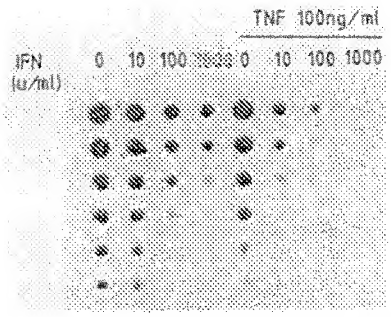

FIG. 5. Synergistic effect of IFN-γ and TNF on the replication of viral DNA. Cells were grown in 60-mm dishes, treated with TNF and IFN-γ at the indicated concentrations for 24 h, and infected with HSV-1 at an MOI of 25 PFU per cell. At 16 h p.i., total DNA was extracted and subjected to hybridization to a nick-translated *Eco*RI TK fragment.

more sensitive to IFN-γ and TNF when a low MOI was employed. However, there was still a significant inhibition in the expression of several HSV-1 proteins, even when high MOIs of HSV-1 were analyzed.

To analyze the effect of TNF and IFN-γ on viral DNA replication, total DNA from infected cells was extracted 16 h after infection. The amount of HSV-1 DNA was quantitated by hybridization with a TK probe. Figure 5 shows that 10 U of IFN-γ per ml alone did not diminish the amount of HSV-1 DNA present in infected cells. However, 100 and 1,000 U of IFN-γ per ml inhibited replication of the HSV-1 genome four- and eightfold, respectively. Although by itself TNF is not inhibitory to HSV-1 DNA replication, the combination of TNF and IFN-γ clearly suppresses viral DNA replication. These results agree well with the findings obtained on viral protein synthesis and suggest that the step blocked by TNF plus IFN-γ is prior to HSV-1 DNA replication.

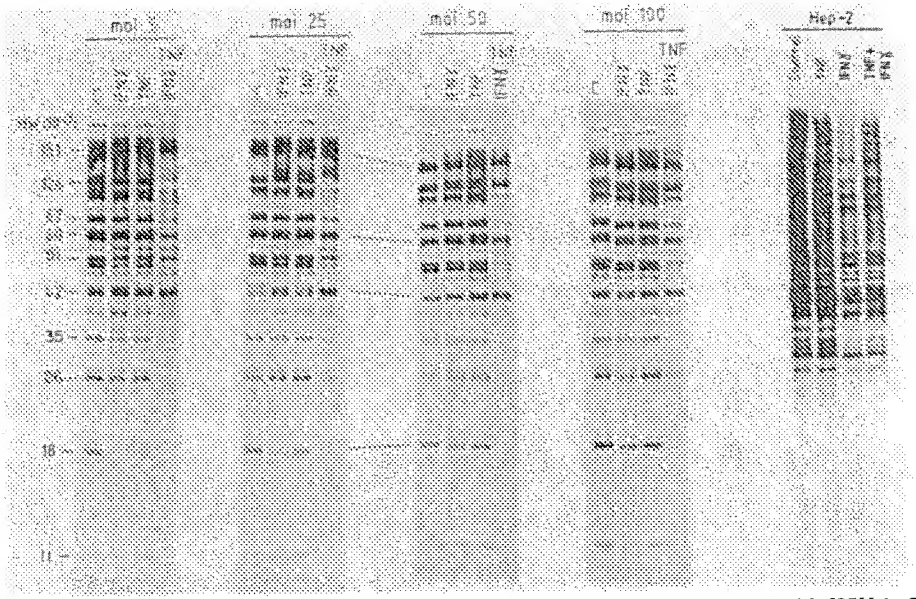

FIG. 4. Synergistic effect of IFN-γ and TNF treatment on protein synthesis in HEp-2 cells infected with HSV-1. Cells were grown in 96-well dishes and treated with TNF (100 ng/ml) and IFN-γ (10 U/ml) for 24 h before infection. Cells were labeled for 2 h with [^{35}S]methionine, and proteins were analyzed as described in Materials and Methods. C, Control. The numbers indicate molecular size in kilodaltons.

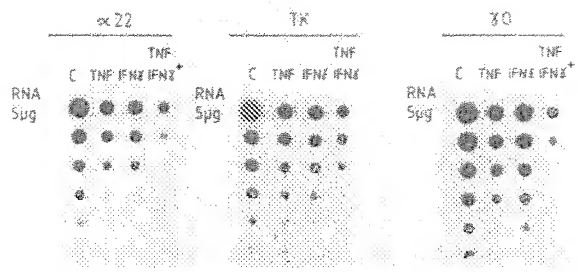

FIG. 6. Synergistic effect of TNF and IFN-γ on transcription of HSV-1 genes in HEp-2 cells. TNF (100 ng/ml) and IFN (10 U/ml) were added 24 h before infection. Conditions of infection, RNA extraction, and hybridization were as described in Fig. 2. C, Control.

The transcription of HSV-1 genes α22, TK, and γ0 was assayed as described above. TNF or IFN-γ used separately had negligible effects on the transcription of early genes α22 (α class) and TK (β class), although both of them when used alone clearly induced some inhibition (Fig. 6). However, the combination of TNF plus IFN-γ prevented the expression of early (α22 and TK) and late (γ0) genes. The conclusion from these results is that TNF plus IFN-γ blocks an early step in HSV-1 replication that comes either at or prior to the transcription of early genes.

To determine whether very early steps in HSV-1 replication, such as HSV-1 binding and entry into HeLa cells, were affected by TNF and IFN-γ, HSV-1 virions were labeled with [^{35}S]methionine, and the entry of these virions into cells was monitored at different times. HSV-1 entered control cells and HEp-2 cells treated with TNF and IFN with similar kinetics and to a similar extent (Fig. 7). The amounts of viral particles adsorbed to uninfected cells at 0°C with and without treatment with TNF and IFN-γ were also similar (results not shown). These results lead us to suggest that HSV-1 is internalized in cells treated with the two cytokines and that the step blocked by them occurs after entry and before or at the level of transcription of HSV-1 early genes.

DISCUSSION

The antiviral mode of action of HuIFN-α against herpesviruses is different from that against other DNA viruses in several respects. First, the production of infectious particles is significantly blocked by HuIFN-α, but viral protein synthesis is not affected (4, 17). The inhibition of virus release (4) and the production of noninfectious viral particles (17) have been documented as the mode of action of HuIFN-α against HSV-1 in human fibroblasts. Our present results point out the differences that exist between the different human IFN species with regard to the inhibitory effects on HSV-1 in HEp-2 cells. Thus, IFN-γ by itself shows some inhibition of viral translation and transcription, particularly when a low MOI is used. Higher doses of HSV-1 are able to overcome the inhibition by IFN. This experiment is of interest because it might help to explain conflicting reports from different groups (4, 6, 15, 17). Our experiments are also consistent with the finding that high IFN-β doses prevent protein synthesis in HSV-1-infected Chang cells (3), because in the HEp-2 system here described, the action of IFN-β is similar to that of IFN-γ rather than to that of IFN-α. Our findings lend additional support to the view that human and mouse IFNs show different effects against HSV-1. Therefore, it is not surprising to us that MuIFN-α-β blocks immediate-early gene transcription in mouse macrophages (5). The conclusion that can be made about the action of IFN against HSV-1 is that it varies according to the system under study in each laboratory (3, 4, 6, 17, 20). Therefore, HSV-1 is rather unique in this regard and differs from picornaviruses and rhabdoviruses, which are more consistently inhibited by high doses of IFN in the different systems analyzed (11, 19). HSV-1 is also at variance with other DNA viruses, such as vaccinia, because it is blocked by the combined action of TNF and low doses of IFN-γ, whereas vaccinia virus translation continues to control levels in cells treated with both compounds (results not shown). Another interesting aspect of the present work is that TNF by itself has no inhibitory effects on HSV-1. This result does not support the idea that TNF stimulates the production of IFN-β_2 in our HEp-2 cells, unless we assume that IFN-β_2 plus TNF has no effect on HSV-1. Experiments are now in progress in our laboratory to clarify this point.

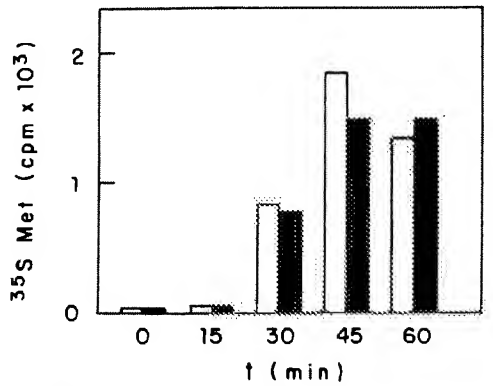

FIG. 7. Effect of IFN-γ and TNF on HSV-1 entry into HEp-2 cells. Entry of radiolabeled virions into untreated cells (white bars) or cells treated with 10 U of IFN-γ per ml and 100 ng of TNF per ml (black bars) was measured as described in Materials and Methods, at the time indicated.

The mode of action of TNF plus IFN-γ against HSV-1 resembles the inhibition by IFN-α-β of HSV-1 in the mouse system. In the murine macrophage model, a blockade in the transcription of the immediate-early genes was observed (5, 6). We have also shown here that the expression of the immediate-early gene α22 is diminished in our system. Keeping in mind that HSV-1 is internalized in cells, there are several possibilities to explain the decrease in α22 expression: (i) the HSV-1 virions enter cells, but they do not reach the nucleus; (ii) decapsidation of HSV-1 virions is not properly achieved; (iii) the HSV-1 DNA is properly located in the nucleus and decapsidates well, but the RNA polymerase does not recognize the promoters of immediate-early genes; and (iv) immediate-early HSV-1 mRNAs are rapidly degraded. At present, we have no convincing evidence to conclude any one of these possibilities. If possibilities iii and iv are true, since neither the transcription of cellular genes nor the stability of cellular mRNAs seems to be affected, we have to propose specific mechanisms acting on viral DNA transcription or viral mRNA stability or both to explain the absence of immediate-early viral mRNA in cells treated with TNF plus IFN-γ.

Much effort has been directed to control HSV-1 and HSV-2 infections in humans by chemotherapy. In fact, either IFN alone or in combination with antiherpes drugs has been described as a potential inhibitor of HSV infections (16, 24). The finding that TNF acts in synergy with HuIFN-γ

opens the possibility of using these two agents in combination against certain HSV-1 diseases.

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EXHIBIT D

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Effect of recombinant hybrid human interferon on replication and morphogenesis of HSV-1 in monkey cells

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Summary

Human recombinant alpha interferon, A/D, significantly reduced the replication and cell fusion induced by herpes simplex virus type 1 in monkey cells. Thin-section electron microscopy of interferon-treated monkey cells showed distinct assembly of nucleocapsids within the nucleus. Analysis of virus-specific proteins by the immunoblot technique confirmed that A/D interferon had no significant effect on the expression of major nucleocapsid proteins, although the expression of glycoproteins B and D was reduced in interferon-treated cells. The possibility of an interferon-induced block at a late stage in virus morphogenesis is discussed.

Human recombinant alpha interferon; Cell fusion; Herpes simplex virus type 1; Glycoproteins B and D; Virus morphogenesis

Herpes simplex virus, types 1 and 2 (HSV-1 and HSV-2 respectively) infections are among the most prevalent encountered by humans and have become prominent targets for potential antiviral agents. Human interferon (IFN) genes, cloned and expressed in bacteria and mammalian cells (Nagata et al., 1980; Streuli et al., 1980; Goeddel et al., 1981; McCormick et al., 1984), have recently received attention because of interferon's potential for treatment of viral infections. These IFNs have been labeled according to three families, alpha, beta and gamma with many

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subspecies varying in amino acid sequences. Furthermore, recombinant DNA experiments have generated hybrid alpha-IFN molecules which show significantly altered biologic properties. A recombinant hybrid human alpha-IFN termed A/D has recently been shown to block lethal infections caused by HSV-2 and encephalomyocarditis virus in mice (Weck et al., 1982; Fish et al., 1983) although the mechanism of action of this IFN was not identified. In addition, three human alpha interferon preparations currently being used in clinical trials, namely rIFN-αA, rIFN-α_2, and lymphoblastoid interferons, had appreciable activity against encephalomyocarditis and vesicular stomatitis viruses in guinea pig transformed and guinea pig embryo cells (Overall et al., 1984). The species specificity of IFN molecules probably is determined by cellular receptors and by the structure of the IFN molecules (Gordon and Minks, 1981). We have previously shown that cloned human alpha-2 and beta-IFNs block HSV replication in human cells at a late stage in viral morphogenesis (Chatterjee et al., 1985). In this communication, we report that recombinant hybrid human alpha-IFN, A/D, significantly inhibited the replication and multinucleate cell (syncytium) formation induced by HSV-1 in monkey cells. Furthermore, we demonstrate that the expression of specific glycoproteins was also greatly affected in these IFN-treated monkey cells, a fact which has not been previously reported. The possibility of future studies in laboratory animals, including primates as model systems is discussed.

In order to determine the effect of IFN A/D on replication of HSV-1 in heterologous system, BS-C-1 (African green monkey kidney) cells were pretreated with 0, 10 and 200 units/ml of this IFN for 18 h and then infected with the MP strain of HSV-1 at a MOI of 1. Supernatant fluids were collected 24 h post-infection and the quantity of virus particles released was determined by plaque assay. Table 1 summarizes the result of this experiment. Both concentrations of A/D IFN significantly ($> 95\%$) inhibited the replication of HSV-1 in heterologous cells. In order to determine whether the observed inhibition in virus replication was due to a block in the expression of specific viral polypeptides which might affect virus assembly and transport, the following experiment was performed. African green monkey kidney cells were pretreated with 0 and 200 units/ml of A/D IFN for 18 h and then

TABLE 1

Effect of A/D IFN on the replication of HSV-1 in monkey cells [a]

IFN (units/ml)	PFU/ml	Percent control
0	3.3×10^5	100
100	1.6×10^4	4.8
200	8×10^3	2.4

[a] BS-C-1 cells, grown as described before (Chatterjee et al., 1985), were pretreated with 0, 100 and 200 units/ml of A/D (Bgl) IFN (specific activity, 1×10^8 units/mg protein; provided by P.W. Trown, Hoffmann-LaRoche Inc., Nutley, New Jersey) for 18 h and then infected with MP strain of HSV-1 as described in the text. Supernatant fluids were collected 24 h post-infection and tested for their ability to form plaques in BS-C-1 cells. Cells were then stained with May-Grunwald-Giemsa as described before (Chatterjee et al., 1985) and the plaque-forming unit (PFU) per ml calculated.

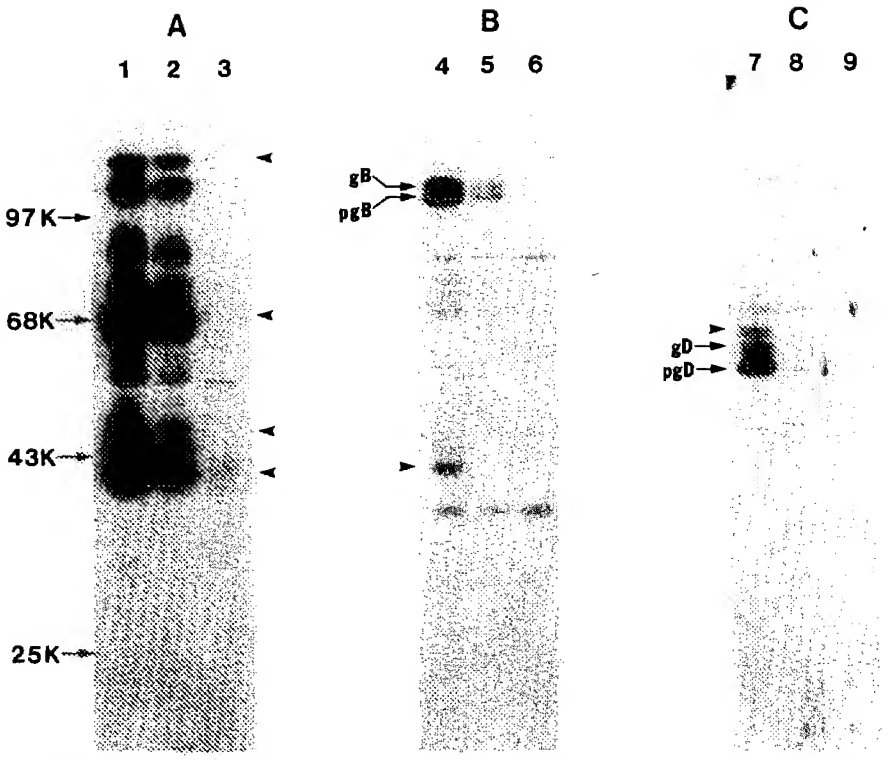
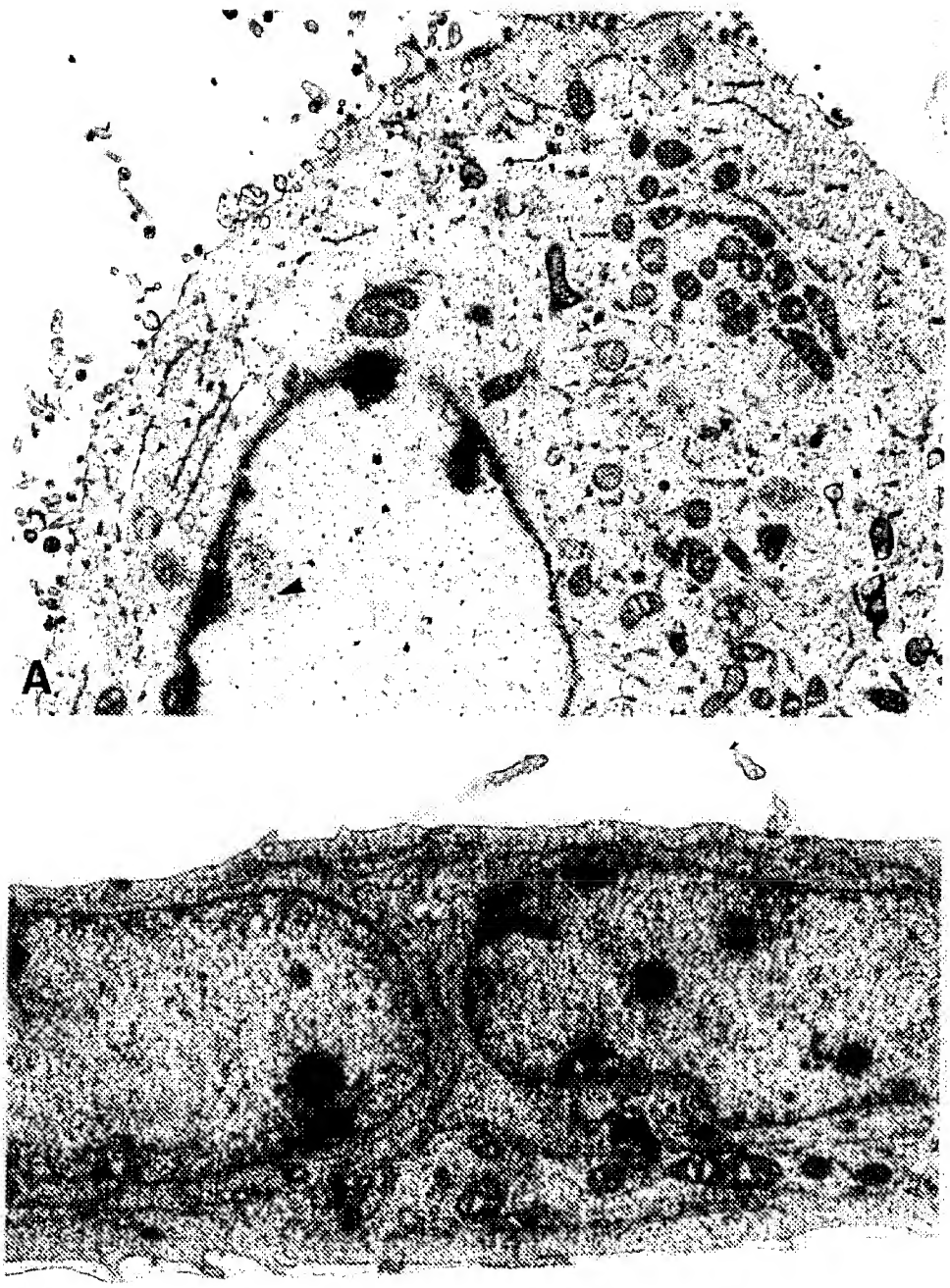


Fig. 1. Effect of A/D IFN on the expression of HSV-1 proteins in IFN-treated and untreated monkey cells. (A) BS-C-1 cells were pretreated with 0 and 200 units/ml of IFN for 18 h and then processed for immunoblotting with rabbit anti-HSV-1 antiserum after infection with MP strain as described in the text. Lane 1, no IFN; lane 2, A/D IFN; lane 3, uninfected control. The major capsid proteins are indicated by arrowheads. (B) Experimental procedure was same as above except the blot was reacted with rabbit anti-gB antiserum. Lane 4, no IFN; lane 5, A/D IFN; lane 6, uninfected control. The pgB denotes the precursor form of the glycoprotein B. Note the faster migrating extra band (arrowhead) in the untreated lane. (C) In this experiment the blot was reacted with rabbit anti-gD antiserum. Lane 7, no IFN; lane 8, A/D IFN; lane 9, uninfected control. The pgD denotes the precursor form of the glycoprotein D. Note the gD-related band (arrowhead) in the untreated lane.

infected with the MP strain as before. Cell lysates collected 18 h post-infection were processed for immunoblotting as described previously (Chatterjee et al., 1985). The blot resulting from this experiment was incubated with rabbit anti-HSV-1 antiserum (provided by B. Norrild, University of Copenhagen, Copenhagen, Denmark; Vestergaard et al., 1977) and finally reacted with [^{125}I]protein A. The result of this experiment (Fig. 1A) demonstrated that this IFN had no significant effect on the expression of major HSV-1 nucleocapsid proteins. In support of the above observation, electron microscopy of virus-infected cells showed formation of distinct nucleocapsids within the nucleus of IFN-treated cells (Fig. 2B). However, in contrast to the untreated cells (Fig. 2A), a very few extracellular mature particles were seen in the IFN-treated cells (Fig. 2B). It should be noted that some of the


A
B

nucleocapsids in the IFN-treated cells lack dense cores. Whether these structures represent an altered or modified form of DNA or entirely lack DNA, remains to be defined. In a preliminary experiment, designed to assay extracellular viral proteins by Western blots, a significant reduction in the release of extracellular particles was noticed (data not shown).

In order to explain the defect in the release of nucleocapsids from the nucleus of the IFN-treated cells, the expression of HSV-1 glycoproteins in these cells was determined by the following immunoblotting experiment. The same IFN-treated and untreated cell lysates used before were analysed for the expression of glycoproteins B and D by using rabbit antiserum against these proteins (provided by B. Norrild). The results (Fig. 1B and C) indicated that, unlike the nucleocapsid proteins, the expression of glycoproteins B and D was reduced by this IFN. However, the reduction of glycoprotein B was not as great as observed with glycoprotein D. Furthermore, in contrast to our earlier observation in human cells (Chatterjee et al., 1985), the glycoprotein profiles were slightly different in monkey cells. The glycoprotein pattern obtained after incubating with antisera against gB and gD displayed additional bands (Fig. 1B and C), which were probably intermediate or degradation products during the post-translational processing pathway as suggested by some investigators (Pereira et al., 1981, 1982; Zezulak and Spear, 1984).

Thus, the block in replication appeared to be at a late stage in viral morphogenesis which was further supported by the fact that this IFN significantly reduced syncytium formation in heterologous cells. In brief, BS-C-1 cells were pretreated with 0 and 100 units/ml of A/D IFN for 18 h and then infected with the MP strain as before. Cells were stained 18 h post-infection and observed for multinucleate cell formation under light microscope. The result of this experiment (Fig. 3) showed that 100 units/ml of A/D IFN almost completely blocked the syncytium formation by HSV-1 in monkey cells (Fig. 3B).

Although earlier studies showed that IFNs were species specific, subsequent development of recombinant hybrid IFN molecules clearly demonstrated a significant level of activity of these molecules in non-human cells (Weck et al., 1981; Rehberg et al., 1982). In support of these observations, we report here that cloned hybrid human IFN A/D can significantly block the replication of HSV-1 in monkey cells. This observed inhibition in replication was not due to any significant reduction in the expression of major nucleocapsid proteins as determined by immunoblotting experiment. Consistent with these observations, electron microscopy also demonstrated the presence of nucleocapsids within the nucleus of IFN-treated cells. A similar situation has also been reported in case of murine

Fig. 2. Electron microscopic observations of A/D IFN pretreated and untreated monkey cells after infection with HSV-1. Electron microscopy was carried out essentially as previously described (Chatterjee et al., 1982a). (A) Thin section of BS-C-1 cells infected with MP strain as before without IFN treatment showing extracellular and intranuclear particles (arrowheads). Magnification × 10,400. (B) Thin section of BS-C-1 cells pretreated with 200 units/ml of IFN and then infected with the same strain of HSV-1. Distinct nucleocapsids can be observed inside the nucleus (arrowheads). Magnification × 17,850.

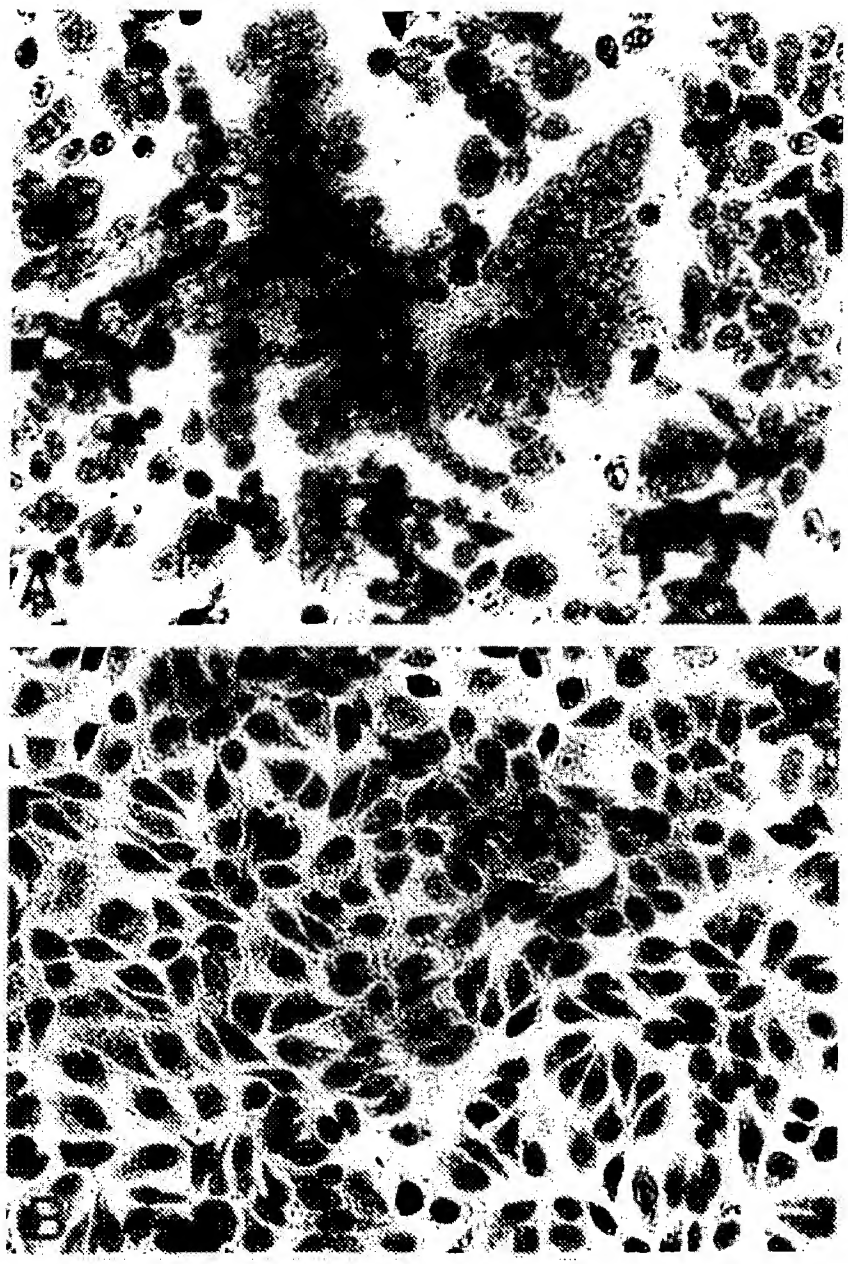


Fig. 3. Effect of A/D IFN on the multinucleate cell formation by HSV-1 in monkey cells. (A) Monolayers of BS-C-1 cells were infected with MP strain as described in the text and then stained with May-Grunwald-Giemsa 18 h post-infection as described before (Chatterjee et al., 1984). (B) In this experiment BS-C-1 cells were pretreated with 100 units/ml of A/D IFN for 18 h and then infected with MP strain and subsequently stained as above.

mammary tumor virus after IFN treatment (Sen and Sarkar, 1980). Thus, the presence of a few extracellular viral particles in IFN-treated cells, coupled with the observation of very little extracellular viral proteins suggest that A/D IFN blocked HSV-1 replication at a late stage in virus morphogenesis, i.e. in the assembly and release of nucleocapsids from the nucleus of the IFN-treated cells. Such a defect in assembly and release could be attributed to the reduced expression of glycoproteins B and D observed in the IFN-treated cells as it is possible that HSV-glycoproteins, like some other viral glycoproteins, are important for assembly and budding of nucleocapsids (Simons and Garoff, 1980; Johnson and Smiley, 1985). It should be noted that the synthesis of vesicular stomatitis virus G protein was inhibited by about 80% in transfected COS cells treated with interferon (Sahni and Samuel, 1986). At the present time, however, the possibility of an effect on other HSV-1 gene products (not quantifiable in our experiments) required for nucleocapsid assembly and budding cannot be ruled out. Alternatively, changes in the membrane fluidity which occur in IFN-treated cells (Pfeffer et al., 1981; Chatterjee et al., 1982b), could also play a role in the defect in morphogenesis. The inhibition in the expression of glycoproteins B and D might explain the significant reduction observed in multinucleate cell formation after IFN treatment. It has been previously reported that both of these glycoproteins are involved in HSV-induced cell fusion (Manservigi et al., 1977; Noble et al., 1983; Kousoulas et al., 1983; Ali et al., 1987). The observation that A/D IFN can significantly inhibit cell fusion in a heterologous system suggests that in vivo therapy with this IFN would prevent virus spread from cell to cell. This information is important because multinucleate cell formation has been considered one mode by which virus can be transmitted from cell to cell (Lodmell and Notkins, 1974). Thus the demonstration that hybrid recombinant human IFNs can prevent replication and cell fusion in heterologous system strongly suggest for an application of these hybrid IFNs in non-human primates as a model system.

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1: J Infect Dis. 1989 Feb;159(2):239-47.

Links

Interleukin-2 protects neonatal mice from lethal herpes simplex virus infection: a macrophage-mediated, gamma interferon-induced mechanism.

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Program in Infectious Diseases, University of Texas, Medical School at Houston 77025.

Administration of human recombinant interleukin-2 (IL-2) protected neonatal mice from a lethal herpes simplex virus (HSV) infection. Protection was not associated with viral antibody production, enhanced natural killer cell cytotoxicity, or intrinsic resistance of macrophages to viral infection. Protection was associated with increased macrophage-mediated antiviral antibody-dependent cellular cytotoxicity (ADCC). Spleen cells from IL-2-treated neonatal mice and from neonatal mice that were treated in vitro with IL-2 transferred protection to neonatal mice. These cells, by adherence, silica, and asialo GM 1 antibody treatment, were shown to be macrophages. IL-2 treatment in vitro enhanced the neonatal macrophages' ADCC function and superoxide release. Similar protection was induced by gamma interferon (IFN-gamma)-treated spleen cells. Antibody to IFN-gamma ablated both IFN-gamma- and IL-2-induced protection by adherent spleen cells. Thus, IL-2-mediated protection against murine neonatal HSV infection was affected by stimulated macrophage activity, via helper T cell-produced IFN-gamma.

PMID: 2492588 [PubMed - indexed for MEDLINE]

Related Links

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Defective production of anti-herpes simplex virus antibody by neonatal mice. Re [illegible]

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Requirement for natural killer cell-produced interferon gamma in defense a [illegible]

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Links

Antiviral activity of a novel recombinant human interferon-alpha B/D hybrid.

Gangemi JD, Lazdins J, Dietrich FM, Matter A, Poncioni B, Hochkeppel HK.

Research Laboratories, Pharmaceuticals Division, CIBA-GEIGY Ltd., Basel, Switzerland.

The antiviral potential of a novel cross-species active, recombinant human interferon-alpha B/D hybrid (rHuIFN-alpha B/D), was evaluated for its efficiacy in cultured human monocytes and in several murine models of viral disease. When examined in 14-day-old human monocyte cultures, rHuIFN-alpha B/D was highly effective in preventing viral replication and cell destruction caused by herpes simplex virus type 1 (HSV-1/VR3). The effect observed with 100 units of this hybrid IFN was as good or higher than that observed with equivalent amounts of rHuIFN-alpha A or IFN-gamma. In addition, a single dose (5 X 10(7) U/kg) of rHuIFN-alpha B/D administered several hours after intranasal infection with HSV-1/VR3 suppressed pulmonary virus replication and prevented death due to interstitial pneumonia. Similarly, mice infected with a more aggressive strain of HSV-1 (McIntyre) were protected when this IFN preparation was administered at the time of virus infection and 1 day later. The anti-retroviral activity of rHuIFN-alpha B/D was examined in two murine leukemia retroviral models, Rauscher (RMLV) and Friend (FMLV), and a murine model of acquired immunodeficiency (LP-BM5). Treatment of RMLV or FMLV infected mice significantly prolonged mean survival times and the number of long-term FMLV survivors. These therapeutic effects were demonstrated when IFN was administered on the day of virus infection or as late as 3 days following infection. Transient reversal of the immunosuppressive effects induced by LP-BM5 infection was observed when rHuIFN-alpha B/D treatment was initiated at the time of virus infection. Moreover, when rHuIFN-alpha B/D was used together with azidothymidine (AZT), the effect of the combination was better than either drug alone.

PMID: 2541210 [PubMed - indexed for MEDLINE]

Protective activity of recombinant cytokines against Sendai virus and herpes simplex virus (HSV) infections in mice

Joji Iida, Ikuo Saiki, Chiaki Ishihara and Ichiro Azuma

The efficacy of recombinant cytokines such as murine interferon-γ (IFN-γ), human granulocyte colony-stimulating factor (G-CSF), mouse granulocytic-macrophage colony-stimulating factor (GM-CSF) and human interleukin-1β (IL-1β) has been examined for augmentation of host resistance against Sendai virus and herpes simplex virus (HSV) infections. All four cytokines were found to protect mice against Sendai virus infection. IFN-γ afforded protection when administered intranasally but not intravenously several days before the infection. Intranasal administration of G-CSF one day before the infection was the most effective administration route and timing. Intranasal administration of GM-CSF was found to afford protection 1 or 3 days before the infection. IL-1β demonstrated therapeutic activity against Sendai virus infection after intranasal administration on the same day as the infection. When each of the cytokines was administered subcutaneously four times daily into cyclophosphamide-treated mice before intravenous infection with HSV, only GM-CSF revealed any protective activity.

Keywords: Sendai virus; cytokines; non-specific host resistance; infection; herpes simplex virus

Introduction

It has been found that host resistance against Sendai virus and herpes simplex virus (HSV) infections can be enhanced by the administration of such bacterial immunoadjuvants as whole mycobacteria, lipopolysaccharides (LPS) derived from Gram-negative bacteria, or muramyl dipeptide (MDP), which is the minimal unit to show immunoadjuvanticity[1–4]. Recently, host resistance to the infections has been shown to be augmented by muramyl tripeptide-phosphatidylethanolamine (MTP-PE), which is a lipophilic derivative of MDP[5]. We have previously reported that N^a-acetylmuramyl-L-alanyl-D-isoglutaminyl-N^ϵ-stearoyl-L-lysine [MDP-Lys(L18)] can protect mice against Sendai virus infection by intranasal administration and that macrophages activated by MDP-Lys(L18) are able to suppress the growth of Sendai virus in the lungs of normal mice[6,7] and can augment host resistance against HSV infection in mice treated with cyclophosphamide (Cy)[7a].

MDP-Lys(L18) is a potent inducer of interleukin-1 (IL-1) among macrophages *in vivo* and colony-stimulating factor (CSF) *in vivo*[8]. Infections of *Listeria monocytogenes*, *Salmonella typhimurium* and ectromelia virus, resistance to which largely depends upon macrophages, have caused the induction of CSF in sera[9–11]. Alveolar macrophages exposed to influenza virus have produced IL-1 *in vitro*[12]. These results suggest that similar factors would be likely to have a role in natural host resistance to microbial infections. Recently, recombinant cytokines such as IL-1, granulocyte CSF (G-CSF), granulocyte-macrophage CSF (GM-CSF) as well as interferon-γ (IFN-γ) have been found to protect mice against *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, *Serratia marcescens* and *Candida albicans* infections in normal or neutropenic mice[13–15].

In this study, we attempted to evaluate the anti-infectious activity of G-CSF, GM-CSF, IL-1 and IFN-γ against Sendai virus infection as well as against HSV infection in Cy-treated mice.

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Materials and methods

Mice

Specific pathogen-free, male inbred Balb/c slc mice were obtained from the Shizuoka Experimental Animal Center and maintained in the Laboratory of Animal Experiment, Institute of Immunological Science, Hokkaido University, under laminar-flow conditions. All mice were used at the age of 4–5 weeks. Water and a pelleted diet (Nihon Nosan Kogyo Co. Ltd, Yokohama, Japan) were supplied *ad libitum*.

Reagent

Recombinant mouse interferon-γ (IFN-γ) (0.7 mg ml^{-1}, specific activity 10^7 U mg^{-1}), prepared by Schering-Plough Corporation, was generously donated by the Suntory Co. Ltd (Osaka, Japan). Recombinant human granulocyte colony-stimulating factor (G-CSF) (50 μg ml^{-1}, specific activity 3×10^7 U mg^{-1}) was kindly supplied by Chugai Pharmaceutical Co. Ltd (Tokyo, Japan). Recombinant human interleukin-1β (IL-1β) (0.1 mg ml^{-1}, specific activity 2×10^6 U mg^{-1}) was kindly supplied by Otsuka Pharmaceutical Co. Ltd (Tokushima, Japan). Recombinant granulocyte-macrophage colony-

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stimulating factor (GM-CSF) (1.17 mg ml^{-1}, specific activity 10^9 U mg^{-1}) was kindly provided by Sumitomo Pharmaceutical Co. Ltd (Osaka, Japan). Those recombinant cytokines were diluted with phosphate-buffered saline (PBS) containing 0.3% bovine serum albumin (BSA). In the preliminary experiment, we observed that the diluent did not have any effect on protection against Sendai virus or HSV infections. N^{α}-Acetylmuramyl-L-alanyl-D-isoglutaminyl-N^{ϵ}-stearoyl-L-lysine [MDP-Lys(L18)] was donated by Daiichi Pharmaceutical Co. Ltd, Tokyo, Japan)

Protection against Sendai virus infection

Details of the methods have been reported previously[6]. The Sendai strain of parainfluenza type I virus was purchased from Flow Laboratories Inc., Rockville, MD. This virus was passaged for 11 generations in suckling C3H/He mice; after the 11th passage the lungs were homogenized in PBS and the supernatant fluid was dispersed in ampoules in 1 ml amounts, frozen and stored as the stock virus suspension at −70°C until use. For each experiment, an ampoule was thawed and 0.03 ml of the virus suspension was administered intranasally under light ketamine (Ketaral-50, Sankyo Co. Ltd, Tokyo, Japan) anaesthesia. When the infectious inoculum was assayed in LLC-MK2 cells (kindly donated by Dr H. Kida, Faculty of Veterinary Science, Hokkaido University), it was found to contain one $10^{4.4}$ haemoadsorption (HAD) unit per 0.03 ml. The survivors were monitored for up to 21 days after the infection. Probability values were calculated by applying the Mann–Whitney U probability test to the mean survival times of the treated group and that of the control group.

Protection against herpes virus infection

Herpes simplex virus (HSV) type 1 strain MacIntyre ATCC VR535 was provided by Dr H. Sakaoka (School of Dentistry, Hokkaido University). A $10^{2.4}$ plaque-forming unit (p.f.u.) of HSV was injected intravenously in the mice which had received intraperitoneally cyclophosphamide (Sionogi Pharmaceutical Co. Ltd, Osaka, Japan) at a dose of 4 mg per mouse one day before the infection. Statistical analysis was performed as described above.

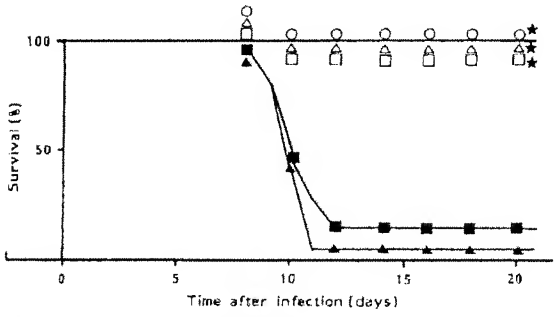

Figure 1 Protective activity of IFN-γ against Sendai virus infection in mice. Seven or 8 Balb/c mice were treated with various doses of IFN-γ 3 days before infection with Sendai virus ($10^{4.4}$ HAD per mouse). ○, 10^3 U (i.n.); △, 10^2 U (i.n.); □, 10 U (i.n.); ■, 10^3 U (i.v.); ▲, control; ★, $p<0.001$

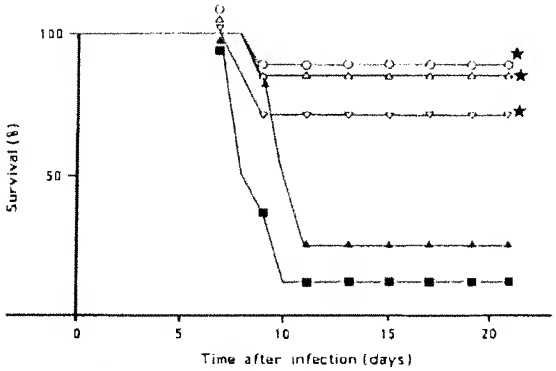

Figure 2 Effect of time intervals between treatment with IFN-γ and infection with Sendai virus. Seven Balb/c mice were given 100 U IFN-γ i.n. on various days before infection with Sendai virus ($10^{4.4}$ HAD per mouse). ○, 5 days before; △, 3 days before; ▽, 1 day before; ■, simultaneously and one day after infection; ▲, control; ★, $p<0.001$

Results

Protective activity of IFN-γ on Sendai virus infection

The mice infected with Sendai virus died with severe pneumonitis 7–15 days after the infection (*Figure 1*). In the first experiment, we compared the protective activity of IFN-γ when administered intranasally (i.n.) or intravenously (i.v.). All the mice that had received 10, 10^2 and 10^3 U IFN-γ i.n. 3 days before infection survived for 21 days after infection, whereas 10^3 U IFN-γ i.v. was not effective (survival rate 13%). The survival rate of the control group was also 13%.

We next examined the effect of the timing of IFN-γ administration on its protective activity against Sendai virus infection (*Figure 2*). Although i.n. administration of 100 U IFN-γ either 5 days, 3 days or 1 day before the infection, showed potent protective activity (88%, 88% and 83%, respectively), postinfection administration (simultaneously with and 1 day after the infection) had no effect (13%). The survival rate of the control group was 25%.

Protective activity of G-CSF and GM-CSF on Sendai virus infection

When the mice were given 2.0 μg G-CSF one day before infection, the survival rate was significantly higher than that of the control group (75%, *Table 1*, Experiment 1). Treatment 3 days before infection showed a slight protective activity (43%) and all the mice (which were injected simultaneously and 1 day after the infection) died within 21 days of infection. Subcutaneous (s.c.) or i.v. administration of 2.0 μg G-CSF 1 day before infection was not effective for protection aginst Sendai virus infection (*Table 1*, Experiment 2). Although the data are not shown, 2.0 μg G-CSF was the minimal effective dose for affording resistance to infection in our experimental conditions. The survival rate of the mice that received 2.0 μg G-CSF s.c. or i.v. 1 day before infection was similar to that of the control group (14%, 14% and 0%, respectively) (*Table 1*, Experiment 2). Intranasal administration of GM-CSF augmented host resistance to infection either 1 or 3 days before infection (*Table 1*, Experiment 3).

Protective activity of rIL-1β on Sendai virus infection

The results presented above show that i.n. administration of IFN-γ and G-CSF before infection afforded a higher rate of protection against Sendai virus infection than i.v. or s.c. administration. Neither simultaneous administration nor postadministration of either cytokine was effective in increasing resistance to Sendai virus infection. As IL-1β has been shown to have some therapeutic effect in controlling microbial infection in mice[13,14], we examined its protective or therapeutic activity against Sendai virus infection by i.n. administration. Pretreatment with 0.2 μg IL-1β either 3 days or 1 day before infection afforded protection against infection (*Figure 3a, b*). Although the simultaneous administration of IL-1β (2 h after infection) was remarkably effective, the post-administration of 0.2 μg IL-1β either 1 day or 3 days after infection was not effective.

Table 1 Protective activity of G-CSF and GM-CSF against Sendai virus infection in mice

Experiment no.	Treatment	Schedule[a] (day)	Route	Survivors/total on day 21	p^b
1	G-CSF	−1	i.n.	6/8	$p<0.001$
		−3	i.n.	3/7	$0.02<p<0.05$
		0, +1	i.n.	0/7	
	Control			0/7	
2	G-CSF	−1	i.n.	3/7	$0.02<p<0.05$
		−1	s.c.	1/7	
		−1	i.v.	1/7	
	Control			0/7	
3	GM-CSF	−3	i.n.	6/6	$p<0.001$
		−1	i.n.	6/6	$p<0.001$
	Control			0/7	

[a]In each experiment, 2.0 μg G-CSF and GM-CSF were administered. Minus values indicate days before infection, plus values are days after infection. [b]Probability values were calculated by the Mann–Whitney *U* test

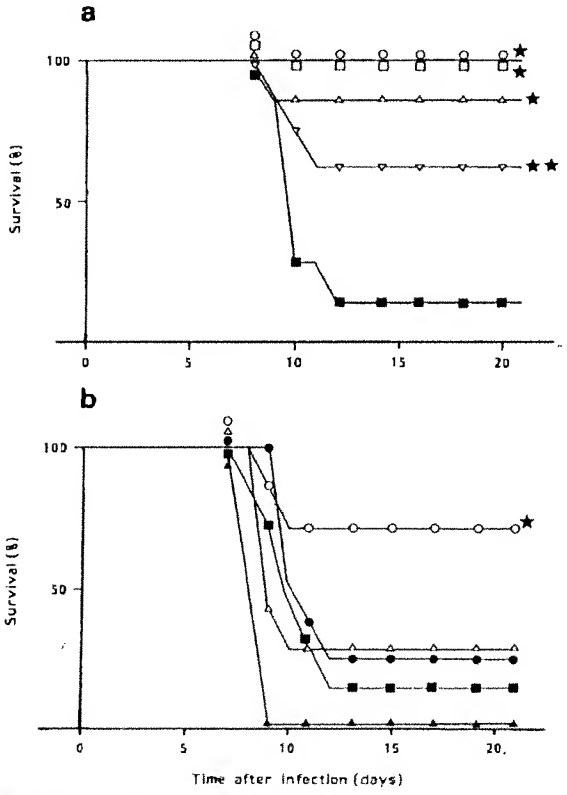

Figure 3 Protective (a) and therapeutic (b) activities of IL-β against Sendai virus infection in mice. Seven Balb/c mice were given 0.2 μg IL-1β i.n. on various days before or after infection with Sendai virus ($10^{4.4}$ HAD per mouse. (a) MDP-Lys(L18) (10 μg) was administered i.n. 1 day before infection. ○, 3 days before, □, 1 day before; △, simultaneously; ▽, MDP-Lys(L18); ■, control; ★, $p<0.001$; ★★, $0.02<p<0.05$. (b) ○, Simultaneously; △, 5 days after; ■, 3 days after; ▲, 1 day after infection; ●, control; ★, $0.02<p<0.05$

Table 2 Protective activity of G-CSF, and IFN-γ against HSV infection in mice which received cyclophosphamide (Experiment 1)

Sample	Treatment[a] On day	Daily dose	No. of survivors/total on day 21	p value[b]
G-CSF	−4, −3, −2, −1	2.0 μg	1/7	
IFN-γ	−4, −3, −2, −1	100 U	3/7	
MDP-Lys(L18)	−3, −1	100 μg	5/7	<0.001
Control			2/7	

[a]Each of the samples was administered s.c. on the indicated days before infection. Cyclophosphamide was injected i.p. 1 day before infection at a dose of 4 mg. Mice were infected with HSV ($10^{2.4}$ p.f.u.) i.v. [b]Probability values were calculated by the Mann–Whitney *U* test

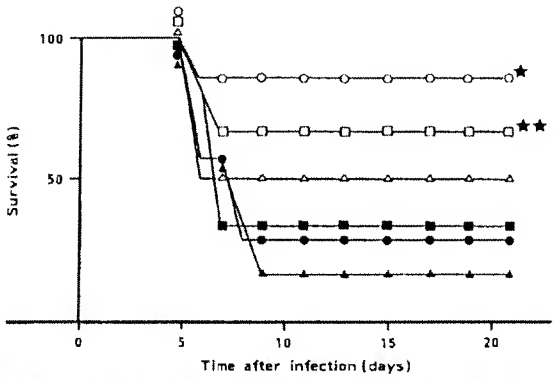

Figure 4 Protective activity of IL-1β and GM-CSF against HSV infection in mice which received cyclophosphamide. Eight BALB/c mice were given IL-1β or GM-CSF subcutaneously 4 days before infection with HSV. MDP-Lys(L18) (100 μg per mouse) was administered s.c. both 3 days and 1 day before infection. Cyclophosphamide (4 mg per mouse) was injected intraperitoneally 1 day before infection. ○, MDP-Lys(L18) (100 μg per mouse); □, GM-CSF (2 μg per mouse); ■, GM-CSF (0.2 μg per mouse); △, IL-1β (0.2 μg per mouse); ▲ IL-1β (0.02 μg per mouse); ●, control; ★, $p<0.001$; ★★, $0.02<p<0.05$

Protective activity of IFN-γ, G-CSF, GM-CSF and IL-1β on herpes simplex virus (HSV) infection in immunocompromised mice

Subcutaneous administration of MDP-Lys(L18), 1 day and 3 days before infection with HSV, almost completely protected the mice that had received intraperitoneally cyclophosphamide one day before the infection (unpublished results). To evaluate the efficacy of IFN-γ, G-CSF, GM-CSF and IL-1β against HSV infection, various doses of the cytokines were administered subcutaneously four times a day before the infection. *Table 2* and *Figure 4* show that MDP-Lys(L18) has a protective action, whereas

IFN-γ, G-CSF and IL-1β were not effective. GM-CSF showed significant protective activity (*Figure 4*).

Discussion

We have already established a model of Sendai virus infection in mice, suitable for application to human pneumonitis caused by influenza virus infection, and we have also reported that the i.n. administration of synthetic adjuvants such as MDP derivatives and chitin derivatives has been found to be a more effective route than i.v., i.p. or s.c. administration[6,7,16]. Our results clearly show that i.n. administration of IL-1β, G-CSF and GM-CSF as well as IFN-γ is an effective protection against Sendai virus infection in mice. The protection afforded by the cytokines used in this study was not attributable to contamination by endotoxins, as the endotoxins in each sample [as assayed by Pyro Dick (Seikagaku Kogyo Co. Ltd, Tokyo, Japan)] were <1 ng ml^{-1}.

The protection afforded by IFN-γ, G-CSF and IL-1β against Sendai virus infection seemed to depend on the route or the timing of administration. Although i.n. administration was effective at a dose of 10 U, intravenous administration of 10^3 U was not effective (*Figure 1*). Intranasal administration of G-CSF 1 day before infection was also effective whereas i.v. or s.c. administration were not (*Table 1*, Experiment 2). Intranasal administration of G-CSF 1 day before infection was effective (*Table 1*, Experiment 1); i.n. administration of GM-CSF was effective when it was administered either 1 day or 3 days before infection (*Table 1*, Experiment 3). These results suggest that i.n. administration of these cytokines is likely to cause an inflammatory response, or that it activates the immune system at the administration site (lungs) and consequently stimulates host resistance against the viral infection. Intranasal administration of MDP-Lys(L18) was able to activate phagocytes in the lungs and the cells were able to suppress the growth of Sendai virus during the early phase of infection[17]. We observed that i.n. administration of IFN-γ was more effective than i.v. administration in activating alveolar macrophages into their cytotoxic state against tumour cells (data not shown). Matsumoto *et al.* have reported that i.p. administration of G-CSF causes a significant increase in the peritoneal exudate cells and leads to the elimination of challenged bacteria in normal or in immunocompromised mice[15]. CSF has been known to play a dual physiological role: it acts both to expand the macrophage-granulocyte population and also to enhance the functional activities of these cells[17]. Subcutaneous administration of IL-1β leads, by its chemoattractive properties, within 1 h to the migration and accumulation of phagocytes around the administration site[18]. These findings suggest that the protection against Sendai virus infection afforded by intranasal administration of IFN-γ, G-CSF, GM-CSF and IL-1β may be attributable to the activation of alveolar macrophages or neutrophils. Only IL-1β showed any therapeutic activity against Sendai virus infection when it was administered simultaneously with the infection (2 h after infection) but it was not effective if it was administered either 1 day, 3 days or 5 days after infection (*Figure 3a, b*). The Sendai virus started to grow in the lung 10 h after infection and reached its maximum titre 2 days after infection[7]. Intranasal administration of IL-1β 2 h after infection may cause rapid accumulation of phagocytes in the lungs before Sendai virus starts to grow. The precise details of the mode of action of IL-1β are now being investigated.

It has been reported that macrophages have a major role in the protection of mice against HSV infection[19–21]. Of the four cytokines used in this study, only GM-CSF showed any significant protective activity against HSV infection in the Cy-treated mice.

In the present study we have attempted to evaluate the efficacy of IFN-γ, G-CSF, GM-CSF and IL-1β as protection against Sendai virus (local infection) and HSV (systematic infection) infections in normal and immunocompromised mice, respectively. The results show that the most effective route for the administration of these four cytokines against the Sendai virus infection is the intranasal route[6,15]. Only GM-CSF protected Cy-treated mice from systematic HSV infection. These results should be of clinical value in the protection of human patients against influenza and herpes virus infections.

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EXHIBIT H

Interleukin-3 protects mice from acute herpes simplex virus infection

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SUMMARY

Evidence presented here from kinetic studies of interleukin-3 (IL-3) production by spleen cells from adult mice infected subcutaneously with HSV-1 and stimulated with virus antigen *in vitro* shows that high levels of IL-3 were produced at the onset of the animal's recovery from the disease state. Injections of anti-IL-3 antibody into HSV-1-infected mice resulted in exacerbation of the disease. Primary mouse embryonic head cells grown in the presence of murine IL-3, when infected with HSV-1, showed a 1000-fold decrease in virus titre compared with untreated control cells. This inhibiting effect was reversed by anti-IL-3 and anti-IFN-α, β and γ antibodies. These data suggest that IL-3 plays a host-protective role against HSV infection and it does so probably by inducing brain cells to produce interferons which then inhibit virus replication.

INTRODUCTION

In both humans and mice herpes simplex virus (HSV), a neurotropic virus, can cause acute viral encephalitis leading to death. However, it is unclear how much of the resultant neuropathology is due to virus cytopathy or to overwhelming immune-mediated disease or a combination of both. The protective effect of T-cell responses against HSV-1 infection has been extensively studied, especially with respect to the role of $CD4^+$ (Chan, Lukic & Liew, 1985; Nash *et al.*, 1987) and $CD8^+$ cells (Nash, Field & Quartery-Papafio, 1980; Nagfuchi *et al.*, 1982; Larsen, Russell & Rouse, 1983; Nash & Gell, 1983; Sethi, Omata & Schneweis, 1983). Macrophages are also generally recognized to play an important role in determining the outcome of a herpetic infection. The difference in susceptibility of different age groups or inbred strains of mice to intraperitoneal infection of HSV correlated with a difference in the ability of macrophages to restrict HSV replication and therefore virus dissemination (Johnson, 1964; Hirsch, Zisman & Allison, 1970; Zisman, Hirsch & Allison, 1970; Stevens & Cook, 1971; Domke *et al.*, 1985). However, less is known of the effect of immune cells on HSV-1-induced neuropathology. Infiltration of lymphocytes, accumulation of macrophages and activation of astrocytes (Adams, 1983; Traugott, Scheinberg & Raine, 1985; Hofman, Von Hanwher & Dinarello, 1986) are constant features of the demyelination seen in multiple sclerosis and certain types of virus-induced encephalitis. While the precise role of these cells in the pathology of HSV-1-induced encephalitis is at present unclear, it is possible that this may be mediated via lymphokines.

Several cytokines have been implicated in determining the outcome of a HSV infection. These include interferon (IFN) (α, b, γ) and interleukin-2 (IL-2) (Domke *et al.*, 1985; Engler *et al.*, 1981; Kohl *et al.*, 1989). Considering the importance of cellular immunity in HSV infection, cytokines that regulate the haemopoiesis of bone marrow stem cells may be as crucial. Among the haemopoietic growth factors, interleukin-3 (IL-3) exhibits pleiotropic effects and can stimulate the proliferation and differentiation of pluripotent progenitor cells that are common to both the lymphoid and hemopoietic lineages (Burgess *et al.*, 1980; Clark-Lewis & Schrader, 1981; Yung *et al.*, 1981; Dexter, 1984). In addition to this, IL-3 can modulate colony-stimulating factor (CSF-1) receptors, thereby affecting proliferation of more mature blood monocytes and tissue-derived macrophages. Pertinent to our interest in HSV-induced encephalitis, is the demonstration that multi-CSF, or IL-3, is a mitogen for amoeboid microglia, the intrinsic brain macrophages (Frei *et al.*, 1986; Giulian & Ingeman, 1988).

The questions we have addressed here are whether IL-3 can protect against HSV-1-induced encephalitis and, if so, by what mechanism. We report here that high levels of IL-3 were produced by spleen and lymph node cells from mice acutely infected with HSV and stimulated *in vitro* with virus antigen, at the onset of the animals recovery from the disease state. Monoclonal anti-IL-3 antibody, when injected into HSV-1-infected mice, can exacerbate the disease. Furthermore, highly purified murine IL-3 markedly inhibited HSV-1 replication in primary mouse embryonic head cell cultures. This inhibition is reversible by anti-IL-3 and anti-IFN-α, β and γ antibodies.

Abbreviations: DLN, draining lymph nodes; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; PB, polymyxin B.

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MATERIALS AND METHODS

Mice

Young adult male CBA mice aged 7–11 weeks, and 17-day-old CBA embryos, were bred at The Wellcome Research Laboratories, Beckenham, Kent, U.K.

Cells

WEHI-3B cells were maintained in RPMI-1640 containing 10% foetal calf serum (FCS) and 5×10^{-5} M 2-ME (Ziltener *et al.*, 1988). The IL-3-dependent line 32DCL was also grown in the same medium but supplemented with WEHI-3B culture supernatant which was prepared from an overnight culture of 5×10^6 cells/ml of serum-free RPMI-1640.

Virus

As previously described (Chan, 1989), stocks of HSV type 1 Krueger strain were prepared from BHK-21/C13 cells, and virus infectivity was determined by a plaque assay using Vero cells.

Antibodies

The anti-IL-3 hybrid cell line 2E11 (IgG1k) was produced and characterized as previously described (Ziltener *et al.*, 1988). The control of anti-Rubella virus monoclonal antibody WRU (IgG1k) was obtained from Wellcome Biotech Ltd, U.K. A polyclonal sheep anti-IL-3 anti-serum and normal sheep serum were also used. Immunoglobulin was precipitated with 45% ammonium sulphate and dialysed extensively against phosphate-buffered saline (PBS) before administration to mice. The antibodies were adjusted to 1 mg/ml and each mouse was injected i.p. 400 μg 4 hr before virus infection and on Days 1, 3 and 5 post-infection. 2E11 was raised in BALB/c mice against synthetic murine IL-3 peptides. It binds to native mouse IL-3 and, when administered *in vivo*, abrogates the increase in splenic mast cells in mice bearing s.c. the IL-3-producing tumour WEHI-3B (Ziltener *et al.*, 1988). The sheep anti-serum can neutralize 1 unit of T-cell-derived IL-3 at 1/50 dilution when assayed against an IL-3-dependent cell line, FDCP1, but has no effect on granulocyte–macrophage colony-stimulating (GM-CSF) factor at the same concentration. Rabbit anti-mouse IFN-α, β and rat monoclonal anti-gamma, were obtained from Lee Biomolecular (San Diego, CA).

Infection of mice

For studies on the effect of anti-IL-3 *in vivo*, male CBA mice aged 7 weeks were infected with $5 \times LD_{50}$ HSV-1 (2×10^6 PFU/mouse) s.c. at 30 μl per hind footpad. Disease symptoms and mortality were recorded daily. Most mortality occurred within 12 days after infection but observations continued until 28 days post-infection. LD_{50} of HSV in mice is highly dependent on age, sex and ambient temperature of the animal house. The value was carefully titred for each set of experiments. Virus clearance was examined by infecting them s.c. with a sublethal dose of HSV-1 in the right pinna (Nash *et al.*, 1980) and virus infectivity remaining in the ear was determined by plaque assay using Vero cells, as previously described (Chan, 1989). Mice were also infected with a sublethal dose of virus s.c. in the footpad and base of tail for kinetic studies on the production of IL-3 by spleen cells or draining lymph node cells *in vitro*.

IL-3 production in vitro

Draining lymph nodes (DLN) or spleens were obtained from CBA mice at various days post-HSV-1 infection. Single-cell suspensions were dispensed at 5×10^6 cells per ml in RPMI-1640 medium without FCS but supplemented with 5×10^{-5} M 2-ME. Cultures with or without heat-inactivated (56°, 1 hr) virus antigen were incubated in an atmosphere of 5% CO_2 at 37° for 24 hr in 24-well Costar plates, and thereafter culture supernatant were collected and stored at −70°.

IL-3 assay

All assays were set up in triplicate in 96-well flat-bottomed tissue culture plates. The IL-3-dependent cell line 32D was washed three times with RPMI-1640 medium (Gibco, Grand Island, NY) and dispensed at 1×10^5 cells per well in 100 μl of RPMI-1640 supplemented with 10% FCS and 5×10^{-5} M 2-ME. Then 100 μl of test culture supernatant were added and cultures were incubated at 37° and 5% CO_2 for 24 hr, pulsed for 18 hr with 1 μCi per well of [^{3}H]thymidine at 5 Ci/mmole (Amersham, Amersham, Bucks, U.K.) and harvested with a cell harvester. IL-3 activity was expressed as [^{3}H]thymidine uptake (c.p.m.). In preliminary experiments, it was established that there was essentially no difference whether IL-3 was assayed on 32DCL or on FCDP2, another IL-3-dependent cell line. IL-3 activity in all the supernatants tested decreased linearly with serial twofold dilutions of the supernatants.

Preparation and infection of mouse embryonic brain cells

Embryos were obtained from pregnant CBA mice killed at Day 17 of gestation. Heads were removed, washed three times with Dulbecco's modified Eagles medium (DMEM; Gibco) minced and transferred in 50 ml DMEM to a 250-ml fluted conical flask containing a small stirring bar and 5 ml of 0·25% trypsin. The flask was then incubated with stirring at 37° for 30 min. Trypsin digestion was stopped by adding 2 ml of FCS and the cell suspension filtered through sterile gauze, washed once in DMEM and resuspended in DMEM containing 10% FCS. The brain cell suspension was dispensed into a 24-well tissue culture plate at 5×10^5 cells per 0·5 ml. A further 0·5 ml of medium with or without appropriate amounts of cytokines and antibody was added to each well. The cells were incubated for 3 days at 37° in 5% CO_2, when another 0·5 ml of medium with or without cytokine and antibody was added and the cells incubated for a further 2 days before infection with 200 PFU of HSV per well. Infection was carried out by removing 1 ml of medium and addition of 0·1 ml virus (200 PFU). The virus was allowed to absorb for 2 hr and then 0·5 ml of medium with or without cytokine and antibody was added and the cells incubated for a further 48 hr before they were harvested by freezing. After another freeze-thaw cycle, the culture supernatants were assayed for virus production by the plaque assay on Vero cells. In some experiments, 1 ng/ml lipopolysaccharide (LPS; Difco Labs, Detroit, ME) with or without 1 μg/ml polymyxin B (PB; Sigma Chemical Co., Poole, Dorset, U.K.) was added as controls. Anti-IL-3 antiserum and control normal serum (final dilution 1/20) were mixed with 100 units purified mouse IL-3 (Genzyme, Kent, U.K.) before addition to the cultures. In preliminary experiments, the virus infecting dose was carefully titrated. The embryonic brain cells are heterogenous and highly susceptible to HSV infection and 200 PFU were found to be most practicable for providing a readout of 4–5 $\log_{10}$ of plaque.

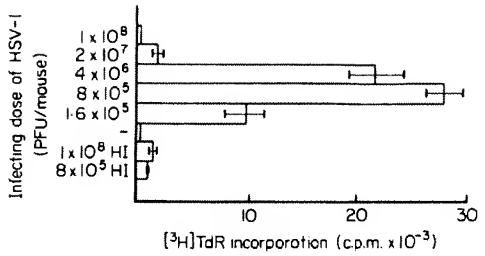

Figure 1. Effect of HSV infecting dose on IL-3 production by spleen cells of CBA mice. Mice were infected in the footpad with graded doses of HSV-1 or heat-inactivated (HI) HSV-1. Spleen cells were harvested 6 days later and stimulated for 24 hr with 7×10^6 PFU of HI HSV-1. IL-3 activity in the culture supernatants was measured as described in the Materials and Methods. Vertical bars = 1 SEM, $n = 3$.

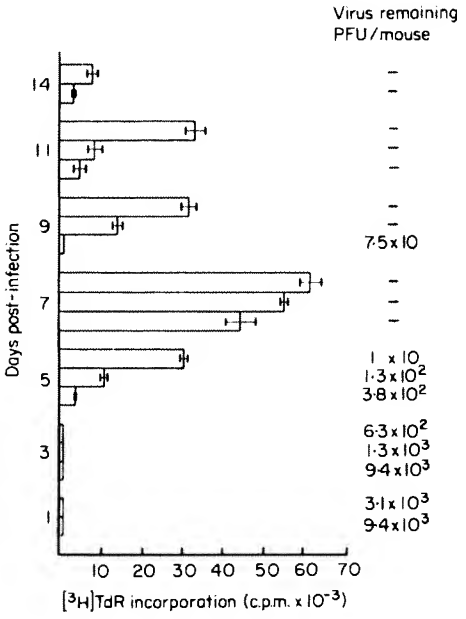

Figure 2. HSV titre and IL-3 activity in individual CBA mice at various times after sublethal infection in the pinna with 3×10^5 PFU of HSV-1. Vertical bars = 1 SEM of triplicate cultures.

The amounts of IL-3 and anti-IL-3 were also carefully titrated in preliminary experiments to obtain a practicable level of neutralization *in vitro*.

Statistical analysis

All experiments were repeated two to three times. Significance was analysed by Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Effects of HSV infecting dose on IL-3 production *in vitro*

Results in Fig. 1 show that the highest level of IL-3 was produced *in vitro* by spleen cells from mice infected with 8×10^5 PFU per mouse of HSV-1. This is a sublethal dose of virus for the age (11-week-old) of the mice used. Little or no IL-3 was produced when lethal doses of virus were used (2×10^7 PFU per mouse). At this dose mice start to die from Day 7. Interestingly, IL-3 production is primed by virus infection but not by inactivated virus. Heat-inactivated HSV (HI-HSV) at the optimal infecting dose of 8×10^5 PFU induced only very low IL-3 production. Even a 100-fold increase of inactivated virus (to 1×10^8 PFU) did not enhance the IL-3 response (Fig. 1).

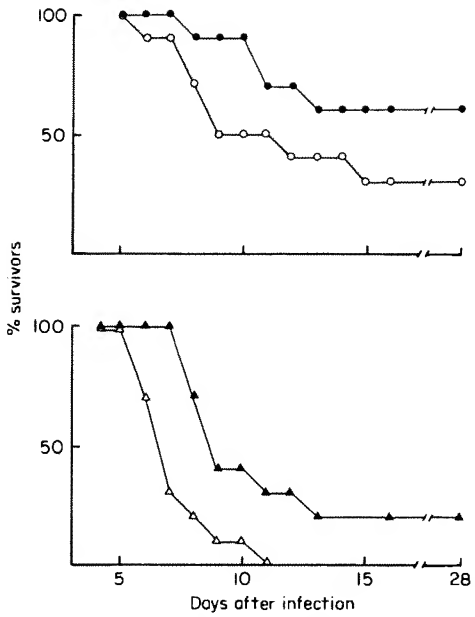

Figure 3. Effects of anti-IL-3 antibody on HSV infection in CBA mice. Groups of 10 mice were infected with $5 \times LD_{50}$ of HSV-1 and injected i.p. with: (a) monoclonal anti-IL-3 antibody (2E11, ○) or a control, non-cross-reacting anti-Rubella antibody (WRU, ●); or (b) polyclonal sheep anti-IL-3 antibody (△) or normal sheep immunoglobulin (▲). The results are representative of four similar experiments. The value for a chi-square statistic on 1 degree of freedom is 3·84 for the 5% level and 6·63 for the 1% level. Thus, there is a significant difference in the average death rate between the treated group and the control group at the 5% level.

Kinetics of *in vitro* IL-3 production

Since IL-3 production by spleen or DLN cells *in vitro* requires infection, we therefore investigated whether the level of IL-3 production varies with the degree of virus clearance from the primary site of infection. While the mice used in Fig. 1 were infected s.c. in the hind footpads, it is experimentally more convenient to monitor virus clearance in the pinna. Figure 2 shows the results comparing virus titre in the ear and IL-3 production by the spleen cells for individual mice over a period of 14 days post-infection. When mice were sublethally infected in the pinna, virus infectivity reached a peak by about Day 4, and was quickly cleared and became undetectable in the pinna by Day 7–9. Virus was cleared from the right pinna of all disease-free mice by Day 7 post-infection. IL-3 was not detectable up to Day 3 post-infection. The level of IL-3 production increased from Day 5 and reached a peak on Day 7 and slowly fell off to low but still significant levels by Day 14. Spleen cells from the mouse with clinical signs of viral encephalitis and with 75 PFU of virus remaining in the ear on Day 9,

Table 1. Effects of IL-3 on HSV-infection of embryo head cells of CBA mice *in vitro**

Material added†	HSV-1 (log_{10} PFU/ml)	P‡
Exp. 1		
Medium	3·98 ± 0·32	—
WEHI-3B	1·96 ± 0·96	< 0·05
IL-3	< 1	< 0·001
Exp. 2		
Medium	3·80 ± 0·25	—
LPS	4·33 ± 0·21	NS
Medium + PB	3·64 ± 0·68	NS
LPS + PB	3·82 ± 0·34	NS
IL-3	1·63 ± 0·45	< 0·01
IL-3 + PB	1·88 ± 1·03	< 0·05
Exp. 3		
Medium	4·53 ± 0·50	—
IL-3 + normal serum	1·85 ± 0·74	< 0·005
IL-3 + anti-IL-3	3·40 ± 0·72	NS
IL-3 + anti-IFN-α, β	5·09 ± 0·21	NS
IL-3 + anti-IFN-γ	4·52 ± 0·10	NS

* For details see the Materials and Methods.

† LPS (1 ng/ml) was used as control for possible contamination of this material in the IL-3 preparation. Polymyxin B (PB) (1 μg/ml) is an inhibitor of LPS. Data for IL-3 + normal serum are pooled results for all control normal sera (anti-IL-3, anti-IFN-α, β and γ).

‡ *P* values compared to medium control; NS, not significant. Each experiment was performed twice with similar results.

produced insignificant amounts of IL-3. Spleen cells from all other mice with clinical signs of viral encephalitis consistently produced little or no IL-3 *in vitro* (data not shown). Thus the overall picture suggests that the production of IL-3 by spleen cells correlates with the onset of virus clearance in HSV-1-infected CBA mice.

Effects of anti-IL-3 antibody on HSV infection in mice

Depending on the age of mice and dose of infecting virus, the kinetics of disease pathogenesis following a lethal s.c. infection are clinically evident by varying degrees of paraplegia, immobility, hunching and incontinence on Days 7–9, with death ensuing in the majority of cases on Days 8–12. Some mice do recover from mild paraplegia. Since increasing levels of IL-3 are produced in mice at the onset of recovery (Fig. 2), anti-IL-3 antibody was administered to the mice at Days 0, 1, 3 and 5 post-infection to determine if the antibody can influence disease development. In a series of experiments it was evident that mice receiving anti-IL-3 monoclonal antibody (2EII) developed exacerbated disease compared to control mice receiving an irrelevant antibody (WRU) (Fig. 3a). Similar results were obtained with the sheep anti-IL-3 antibody (Fig. 3b). The anti-IL-3 antibody-treated mice developed disease symptoms earlier, starting at Day 5, with mice dying from Day 6 instead of Day 8 in the controls. Mortality at the end of 28-day period was twice as many as in the control group.

Effects of IL-3 on HSV-1 infection of embryo brain cells *in vitro*

Since the pathology of the disease lies in the central nervous system, embryo brain cells of CBA mice were used to examine the *in vitro* effect of IL-3 as a regulator of pluripotent progenitor stem cells. IL-3 may act directly on the development of microglial or indirectly on HSV-1 replication in neuronal and glial cells. Primary mouse embryonic head cells grown in the presence of murine IL-3 show a 1000-fold decrease in virus titre compared with untreated control cells or cells treated with LPS ± PB (Table 1). The lack of effect of deliberately added LPS shows that the decrease in virus titre is not due to contaminating concentrations of LPS in the IL-3 preparation. This is confirmed by the presence of PB (an inhibitor of LPS), which has no effect on the activity of the IL-3 used. When anti-IL-3 antiserum was mixed with IL-3 before addition to the cultures, the inhibition of virus replication was reversed compared with cultures treated with normal serum. The inhibitory effect of IL-3 can also be reversed by anti-IFN-α, β and anti-IFN-γ antibody (Table 1, Exp. 3). The anti-IFN antibodies had no effect on IL-3 activity when assayed on an IL-3 dependent cell line (data not shown).

DISCUSSION

In previous experiments it has been shown that during acute HSV-1 infection the neuropathology induced in the brain includes demyelination and accumulation of macrophages and amoeboid microglia in the brain parenchyma and also as perivascular infiltrates that express the CD4 antigen (Chan, Javanovic & Lukic, 1989). The change in microglial morphology from ramified to amoeboid and the coincidental increase in CD4 is also evident after inflammation and changes in the blood–brain barrier (Perry & Gordon, 1987). Therefore the accumulation of macrophages following HSV infection of brain cells may be due to an invasion and proliferation of blood-derived monocytes and/or activation and proliferation of microglial cells, induced perhaps by an influx of immunomodulators like the colony-stimulating factors. Since IL-3 can act as a growth factor for microglial cells (Yung *et al.*, 1981), this led to the question of whether IL-3 protects against HSV-1 infection via its effect on microglial cells.

While macrophages have long been implicated in the inhibition of virus replication and hence resistance to HSV infection in mice (Johnson, 1964), it was only recently that cytokines were shown to be involved. Using the mouse model of intraperitoneal (i.p.) infection, Engler *et al.* (1981) demonstrated that there is a marked increase of IFN (α, β, γ) production with resultant increase in natural killer (NK) cell activity in peritoneal exudate cells from HSV-infected mice. In addition, Domke *et al.* (1985) demonstrated that IFN (α, β, γ) can directly inhibit virus replication at an early step prior to or during the synthesis of early viral proteins and that IFN-γ act synergistically with IFN-α or IFN-β. Recently, Kohl *et al.* (1989) showed that injection of human recombinant IL-2 or IFN-γ protected neonatal mice from a lethal HSV infection. Since antibody to IFN-γ ablated protection induced by either of the two lymphokines, they postulated that IL-2 mediated protection by stimulating macrophage activity, via T-cell-produced IFN-γ. Since murine bone marrow-derived macrophages have been shown to release IFN when exposed to CSF-1 (Fleit & Rabinovitch, 1981; Moore *et al.*, 1984), it is conceivable

that microglia may release IFN when exposed to IL-3. This possibility is clearly supported by results shown in Table 1. However, the culture supernatant of head cells treated with IL-3 contained only low levels of IFN which were barely detectable by a radioimmunoassay or a cytopathic assay (data not shown). It may be that the amounts of IFN induced by IL-3 were low but effective against HSV in a microenvironment.

The priming of IL-3 production paralleled that of the onset of virus clearance from the peripheral site of infection. This in turn correlated with an increase in T-cell activity in response to HSV infection. It has been reported that astrocytes produce an IL-3-like activity (Frei *et al.*, 1986). However, this has not been demonstrated by molecular analysis or Northern blotting. T cells play a crucial role in protection against HSV (Nash *et al.*, 1987, 1980; Nagfuchi *et al.*, 1982; Larsen *et al.*, 1983; Nash & Gell, 1983; Sethi *et al.*, 1983) and, at present, the most likely source of IL-3 in the brain of HSV-infected mice appears to be specifically activated T cells. However, when mice were lethally infected (Fig. 1) or showed clinical signs of viral encephalitis (Figs 2 and 3), only low levels of IL-3 were produced *in vitro*. It appears that while live virus infection is required to induce IL-3 release by activated T lymphocytes (Fig. 1), a debilitating dose of virus on the other hand would have the opposite effect. The lack of IL-3 production in this case may result from an incapacitation of the immune response, since T lymphocytes which normally are not permissive to HSV replication may become infected on activation by IL-2 (Braun & Kirchner, 1986).

While administration of monoclonal anti-IL-3 antibody to mice infected with HSV exacerbated disease, suggesting that the presence of IL-3 can protect against HSV infection, the reverse effect was seen when mice infected with *Leishmania major* (Feng *et al.*, 1988) or *Plasmodium berghei* (Grau *et al.*, 1988) were simultaneously injected with anti-IL-3 antibody alone or, in the latter situation, together with anti-GM-CSF antibody. In these cases, IL-3 appears to exacerbate disease development. Thus IL-3 has pleiotropic effects and, depending on the pathogenesis of a disease, IL-3 can either protect or exacerbate.

Though the evidence obtained with the administration of anti-IL-3 antibody is indirect, we did not inject IL-3 into mice *in vivo* because of its short half-life and because the use of diffusion chambers only produces a localized concentration. Therefore IL-3 was added to head cell cultures to study its effect *in vitro*.

Head cell cultures containing a heterogeneous population of cells were used to simulate the *in vivo* situation. Addition of IL-3 to the cultures reduced the virus yield by 10^3 PFU per ml compared with control cultures grown in the medium alone or in the presence of LPS or PB. Thus the reduced virus yield is not due to the mitogenic effect of contaminating concentrations of LPS in the IL-3 preparation used. Addition of anti-IL-3 antibody, on the other hand, reversed the effect of IL-3. Furthermore, the inhibition of HSV replication by IL-3 was also completely abolished by anti-interferon antibodies which have no direct effect on IL-3 activity. Our results therefore demonstrate that IL-3 plays a protective role against HSV-1 infection in the mouse. It does so probably by acting on brain cells to produce other cytokines such as IFN-α, β and γ, which then inhibit virus replication. The identity of the brain cells involved in the present system is unclear. It may require the participation of a number of different cell types, some of which respond directly to IL-3. Fibroblasts and lymphocytes appear not to be the targets of IL-3, since IL-3 receptors have only been found on cells of the monocytic lineage (Ziltener *et al.*, 1988). The detailed mechanism of IL-3-mediated inhibition of HSV replication, as well as the cell types involved is currently under investigation.

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EXHIBIT I

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Archives of Virology

Recombinant Interleukin-1α, Interleukin-2 and M-CSF-1 enhance the survival of newborn C57BL/6 mice inoculated intraperitoneally with a lethal dose of herpes simplex virus-1

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Summary. Recombinant Interleukin-1α (IL-1α), Interleukin-2 (IL-2) and recombinant macrophage colony-stimulating factor-1 (M-CSF-1) as well as combinations of IL-2 and M-CSF-1 were studied for their ability to protect seven-day-old C57BL/6 mice against HSV-1 infection. Treatment of the mice with IL-2, M-CSF-1 or combinations of IL-2 and M-CSF-1 significantly increased survival rates. Treatment with IL-1α (10 U and 100 U/mouse) was most effective in protection against HSV-1, resulting in significantly increased survival rates more than four times greater than the survival rate of the infected control group.

Introduction

Disseminated herpes simplex virus-1 (HSV-1) infection of newborns is a severe condition with mortality reaching as high as 15–20% in human neonates [13]. The outcome of HSV-1 infection can be influenced by the immunocompetency of the host. Defects in the immune system of neonates, possibly resulting in increased susceptibility to HSV-1 infection, have been identified both in humans and in the murine model. In the latter, most studies have been conducted with C57BL/6 mice which, at the age of four weeks, are resistant to intraperitoneal infection with HSV-1 as adults, but as newborns are highly susceptible [15, 32, 41]. Human studies suggest a delayed production of anti-HSV-1 antibody in neonates with low antibody-dependent cellular cytotoxicity (ADCC) as compared to adults [18]. The murine model reveals that newborn mice also have poor antibody responses to exogenous antigens [22]. However, administration of large doses of anti-HSV-1 antibodies immediately before or after infection with HSV-1 may alter the result of the infection [1, 3, 8, 26, 34]. Other immunologic defects previously described in newborn mice include defective macrophage function and impaired T-cell function, characterized by altered lym-

phokine production [14, 17]. Clearance of the virus from the peritoneum following intraperitoneal (i.p.) infection is accomplished largely by peritoneal macrophages [34]. The state of activation [2] and differentiation [35] of macrophages affects their ability to restrict HSV-1 replication in vitro [31]. When mature macrophages are absent from the peritoneum, the virus is able to penetrate the central nervous system, resulting in a lethal encephalitis in the mouse model [42]. Macrophages of neonate animals are unable to restrict viral replication [17]. Numerous attempts have been made to overcome these immunological defects. The transfer of peritoneal cells from nonimmune syngeneic adult mice to newborns results in reconstitution of the ability to produce antibodies [19]. This appears to be due to both macrophages and helper T-cell populations although the latter may be replaced with soluble helper T-cell products [19]. Lethal HSV-1 infection may be prevented either by administration of macrophages and T-cells or macrophages and T-cell-lymphokine-containing fraction [14]. T-cells may also be replaced with human recombinant interleukin-2 (IL-2) [14].

IL-1 plays an important role in local and generalized inflammatory and immune responses and has a wide spectrum of biological activities, including the induction of T lymphocyte proliferation and the initiation of the acute phase response [5, 6]. It also stimulates the production of IL-2, IL-3, IL-6 and the interferons [6] and acts in the augmentation of the immune response to antigens. Although originally described as a product of activated phagocytic cells, studies have shown that it is synthesized by numerous cell types. Expression of the IL-1 gene is induced in the course of antigen presentation to T-cells [21]. IL-1 activity is encoded by two different genes, IL-1α and IL-1β. The murine cDNAs were cloned and shown to detect RNA species of 2.1 and 1.4 kb, respectively, by the Northern blot technique [9, 24]. Both species share the same range of biological activities [4, 6, 36] and bind to the same 80 kDa receptor [7].

IL-2 is secreted by T lymphocytes upon stimulation with mitogen or antigen and has effects on several immune functions including enhancement of natural killer (NK) cell activity, the induction of lymphokine-activated killer (LAK) cells and stimulation of interferon-γ production [40]. It also stimulates antiviral cytotoxicity of both adult and neonate human cells [20]. Human recombinant IL-2 (rIL-2) has previously been shown to protect against acute HSV-2 genital infections in guinea pigs [40] and is an effective immune therapy in neonatal mice when administered one day prior to infection [16]. Furthermore, the production of fully differentiated T-cells has an absolute requirement for IL-2. Resting T-cells do not make IL-2 nor do they respond to external sources of the factor. Both stimulation of IL-2 production and display of the IL-2 receptor requires the introduction of antigen.

Macrophage colony-stimulating factor (M-CSF-1) belongs to a group of growth factors that stimulate proliferation and differentiation of bone marrow progenitor cells and may also stimulate mature cells. Its primary role is macrophage activation [39]. Although IL-2 protection of newborn mice from HSV-

1 infection was reported [16, 17], no such studies were reported on the effect of M-CSF-1 on the resistance of newborn mice to HSV-1 infection. Since macrophages are known to possess receptors for both M-CSF-1 and IL-2 [11, 12], it is therefore possible that both cytokines might act to stimulate macrophage function. A combination of both cytokines may act synergistically to protect neonatal mice against lethal HSV-1 infection.

With the known interaction of various cytokines in the induction of the immune response in mind, we undertook the study of the protective effects of various concentrations of the recombinant cytokines IL-1α, IL-2 and M-CSF-1 and combinations of IL-2 and M-CSF-1 on the outcome of lethal HSV-1 infection in 7-day-old C57BL/6 mice.

Materials and methods

Virus

Herpes simplex virus type 1 (HSV-1) KOS strain was obtained from Prof. F. Rapp, Pennsylvania State University, Hershey, U.S.A. The virus was propagated in BSC-1 cell cultures, grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% calf serum (Beth Haemek, Israel). Mice were injected intraperitoneally (i.p.) with either 10^3 or 10^4 pfu/mouse. Under these conditions, most deaths occurred 5 days post infection (p.i.) and tapered off by day 10 in most cases.

Mice

C57BL/6 mice, aged 7 days at the start of the experiment were obtained from the Hebrew University animal facilities in litters of 10 pups. Each litter served as one experimental group. The total number of experiments with each experimental test group is indicated in Table 1.

Cytokines

IL-1α, highly purified recombinant human interleukin-1 from *E. coli* containing 5×10^4 U/ml and > 95% pure by SDS-PAGE was purchased from Genzyme Corporation (Boston, MA). IL-2 (lot LSP-805), a highly purified recombinant human interleukin-2 from *E. coli* [28, 37] containing 18×10^6 international units/ml, was 99% pure by SDS-PAGE and contained 0.012 ng/ml endotoxin by the limulus amoeba lysate assay. M-CSF-1 (lot DP-403), a highly purified recombinant human macrophage Colony-Stimulating Factor [10, 23] containing 1.24×10^8 U/ml, was > 95% pure by SDS-PAGE and contained < 0.01 ng/ml endotoxin. These cytokines were generously provided by Cetus Corporation (Emeryville, CA). Dilutions of IL-2 and M-CSF-1 were carried out in DMEM and IL-1α was diluted in sterile PBS containing 0.1% FCS as carrier protein. All cytokines were injected into the mice intraperitoneally (i.p.) at concentrations as indicated in Table 1.

Statistical analysis

Results of experiments were analyzed by χ^2 testing. Results were considered significant if $p < 0.05$.

Determination of virus in mouse brains

Brains of mice that died during the experiment were removed, homogenized in 1 ml DMEM, sonicated for 1 min and serially diluted in DMEM for titration on monolayers of BSC-1

cells in 12-well plates (Falcon). After adsorption for 1 h, monolayers were overlayed with 2% agar and 2× DMEM (1 : 1 vol). After incubation for 3 days at 37 °C in a humidified atmosphere enriched with 5% CO_2, monolayers were fixed in 25% formaldehyde and stained with crystal violet.

Results

Seven-day-old C57BL/6 mice were injected intraperitoneally with IL-1α, IL-2, M-CSF-1 or a combination of IL-2 and M-CSF-1 and were infected one day later with either 10^3 pfu/mouse or 10^4 pfu/mouse KOS strain HSV-1. Survival of the mice was recorded up to 21 days p.i.

Protective effect of IL-1α

The results in Table 1 (mouse group 1) show that infection of untreated one-week-old C57BL/6 mice with 10^3 pfu/mouse of the KOS strain of HSV-1 resulted

Table 1. Influence of cytokine treatment on survival rate of newborn mice infected with HSV-1 (10^3 pfu/mouse)

Mouse group	Treatment	No. of experiments[a]	Survival		p-value[c]
			surv./total[b]	%	
1	Control: HSV-1 KOS	3	6/30	20	
2	10 U IL-1α[d]	2	18/20	90	< 0.0001
3	100 U IL-1α[d]	3	29/30	97	< 0.0001
4	1000 U IL-1α[d]	1	3/10	30	0.51
5	Control: HSV-1 KOS	5	16/48	33	
6	6×10^3 U IL-2[e]	4	23/38	61	0.0119
7	6×10^4 U IL-2[e]	5	27/50	54	0.0393
8	10^3 U M-CSF-1[f]	4	21/39	54	0.0543
9	10^4 U M-CSF-1[f]	4	9/38	24	0.33
10	10^5 U M-CSF-1[f]	3	12/25	48	0.22
11	10^6 U M-CSF-1[f]	2	6/16	38	0.76
12	10^6 U M-CSF-1 × 2[g]	2	6/19	32	0.89
13	6×10^3 U IL-2 + 10^3 U M-CSF-1[h]	2	15/20	75	0.0017
14	6×10^4 U IL-2 + 10^6 U M-CSF-1[h]	4	30/39	77	0.0001

[a] In each experiment one litter (10 newborn mice) was treated with the cytokines and subsequently inoculated with HSV-1 (KOS) at 10^3 pfu/mouse

[b] Total number of survivors on day 21 p.i. per total number of experimental animals in each group

[c] χ^2 tests (groups 2 to 4 relative to control group 1, groups 6 to 14 relative to control group 5)

[d] Animals received single doses of IL-1α as indicated 24 h prior to virus infection

[e] Animals received single doses of IL-2 as indicated 24 h prior to virus infection

[f] Animals received single doses of M-CSF-1 as indicated 24 h prior to virus infection

[g] Animals received single doses of M-CSF-1 as indicated

[h] Animals received single doses of IL-2 and M-CSF-1 as indicated 24 h prior to virus infection

in a survival rate of 20% at 21 days p.i. Treatment of the neonates with IL-1α at doses of 10 U/mouse or 100 U/mouse resulted in a highly significant increase in survival rates to 90% (mouse group 2, $p < 0.0001$) and 97% (mouse group 3, $p < 0.0001$), respectively. IL-1α at doses of 1000 U/mouse resulted in a survival rate of 30% (mouse group 4, $p = 0.51$). These results are presented graphically in Fig. 1 A. Statistical comparison of the survival rate obtained after treatment with 10 U/mouse (mouse group 2) versus 100 U/mouse (mouse group 3) IL-1α shows $p = 0.33$, an insignificant difference (Table 2). However, comparison of mouse group 2 (10 U/mouse IL-1α) with group 4 (1000 U/mouse IL-1α) or group 3 (100 U/mouse IL-1α) with group 4 reveals highly significant p values of $p = 0.0007$ and $p < 0.0001$, respectively.

Protective effect of IL-2

The results for mouse group 5 (another set of controls) show that infection of untreated one-week-old C57BL/6 mice with 10^3 pfu/mouse of the KOS strain of HSV-1 resulted in a survival rate of 33% at 21 days p.i. Treatment of the neonates with either 6×10^3 U/mouse or 6×10^4 U/mouse of recombinant IL-2 (rIL-2) one day prior to infection (mouse groups 6 and 7, repeated 4 and 5 times respectively) resulted in a statistically-significant increase in survival with survival rates of 61% ($p = 0.0119$) and 54% ($p = 0.0393$), respectively (represented in Fig. 1 B). The difference in survival rates between mouse groups 6 and 7 was not significant ($p = 0.64$).

Protective effect of M-CSF-1

Treatment with 10^3 U/mouse recombinant human M-CSF-1 (group 8, repeated four times) also resulted in a significant increase in survival to 54% ($p = 0.0543$), as compared to the survival of the untreated infected controls (group 5) (Fig. 1 C). Treatment with M-CSF-1 at doses of 10^4 U/mouse or 10^6 U/mouse (mouse groups 9, 10 and 11) or with two individual doses of M-CSF-1 10^6 U/mouse one day prior to and one day after infection (mouse group 12) showed no significant difference in survival rates relative to the control. In fact, administration of 10^4 U/mouse M-CSF-1 had an apparently deleterious effect, decreasing survival from 33% to 24% (mouse group 9). The reason for this effect is not known.

Protective effect of combinations of IL-2 and M-CSF-1

Injection of both IL-2 and M-CSF-1, administered one day prior to infection (mouse groups 13 and 14) significantly increased survival rates more than two-fold (to 75% survival, $p = 0.0017$), with no significant difference between a combination of 6×10^3 U/mouse IL-2 and 10^3 U/mouse M-CSF-1 (mouse group 13) or one of 6×10^4 U/mouse IL-2 and 10^6 U/mouse M-CSF-1 (mouse group 14) ($p = 0.87$) (results are represented graphically in Fig. 1 D). While a statistical comparison of the increase in survival rates between groups receiving 6×10^3

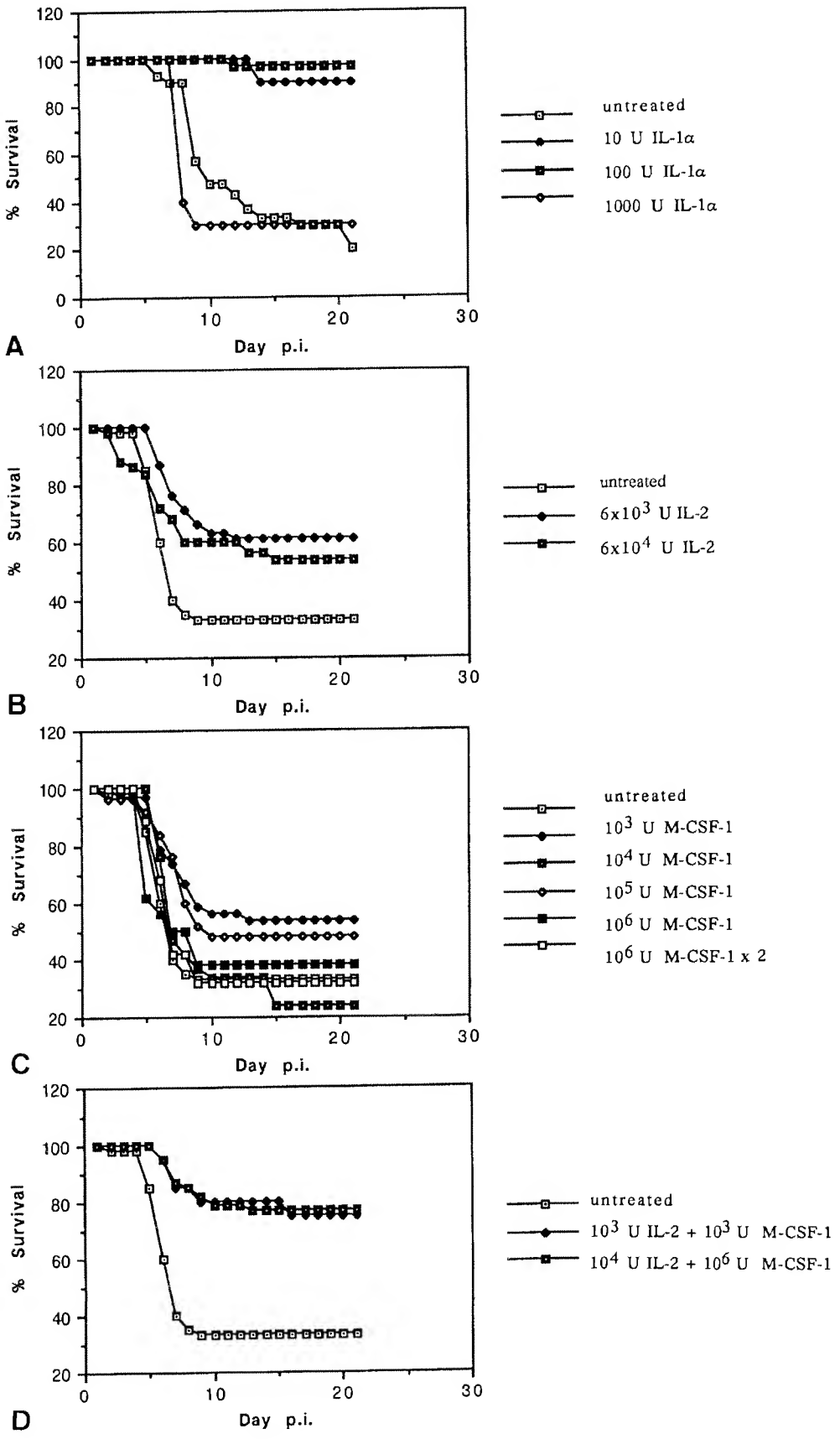
% Survival
Day p.i.
A
untreated
10 U IL-1α
100 U IL-1α
1000 U IL-1α
B
untreated
6x10^3 U IL-2
6x10^4 U IL-2
C
untreated
10^3 U M-CSF-1
10^4 U M-CSF-1
10^5 U M-CSF-1
10^6 U M-CSF-1
10^6 U M-CSF-1 x 2
D
untreated
10^3 U IL-2 + 10^3 U M-CSF-1
10^4 U IL-2 + 10^6 U M-CSF-1

Table 2. Statistical comparison of survival rates of newborn mice pretreated with cytokines and infected with HSV-1

Mouse group[a]	Treatment	Survival		p-value[b]
		surv./total	%	
2	10 U IL-1α	18/20	90	0.33
3	100 U IL-1α	29/30	97	
2	10 U IL-1α	18/20	90	0.0007
4	1000 U IL-1α	3/10	30	
3	100 U IL-1α	29/30	97	< 0.0001
4	1000 U IL-1α	3/10	30	
6	6×10^3 U IL-2	23/38	61	0.27
13	6×10^3 U IL-2 + 10^3 U M-CSF-1	15/20	75	
7	6×10^4 U IL-2	25/50	54	0.025
14	6×10^4 U IL-2 + 10^6 U M-CSF-1	30/39	77	

[a] Mouse groups same as in Table 1
[b] χ^2 tests

IL-2 alone or a combination of 6×10^3 IL-2 and 10^3 U M-CSF-1 reveals an insignificant difference ($p = 0.27$) (Table 2), a comparison between the groups which received 6×10^4 U IL-2 and those which received a combination of 6×10^4 U IL-2 and 10^6 U M-CSF-1 shows $p = 0.025$, a moderately significant difference.

The effect of cytokines on the outcome of infection of one-week-old C57BL/6 mice with 10^4 pfu/mouse of the KOS strain of HSV-1 was also studied. This virus dose killed all the infected, untreated newborn mice. Treatment of the newborn mice with rIL-2 at 6×10^4 U/mouse prior to infection resulted in a highly significant increase in survival to 70% of the mice ($p = 0.0000$, to four decimal places) while M-CSF-1 at 10^3 U/mouse protected 30% of the mice ($p = 0.008$). A combination of rIL-2 at 6×10^4 U/mouse and M-CSF-1 at 10^6 U/mouse protected 60% ($p = 0.001$) of the mice. These results show that cytokine treatment is of protective value against a highly lethal dose of HSV-1.

Fig. 1 A–D. Effect of cytokine treatment on the survival of newborn C57BL/6 mice infected with HSV-1. Mice were injected intraperitoneally with various doses of cytokines 24 h before intraperitoneal infection with 10^3 pfu/mouse of HSV-1 (KOS strain). Results are expressed in terms of percent survival. **A** Protective effects of IL-1α treatment. **B** Protective effects of IL-2 treatment. **C** Protective effects of M-CSF-1 treatment. **D** Protective effects of combined cytokine treatment

Fate of virus in cytokine-treated mice

Virus content in brains from those newborn mice that were infected with HSV-1 and died during the observation period of 21 days was determined. Virus plaques were isolated from brain homogenates of the majority of infected newborn mice that succumbed to the virus infection, indicating that viral encephalitis was indeed the cause of death. Virus titres as high as $10^3 - 10^4$ pfu/ml of brain suspension were recorded. This result is consistent with the observation of hind leg paralysis in many mice prior to death, indicating that the infecting virus penetrated the adrenal gland and the spinal cord and also infected the central nervous system [27]. No infectious virus was found in the brains of the HSV-1-inoculated neonatal mice that were protected by cytokines and survived the virus infection.

Discussion

The increased susceptibility of neonate animals and humans to lethal infection with HSV-1 may be due to a series of immunologic defects including defective macrophage function (with macrophages unable to process antigens) and impaired T-cell function. The role of T-cells may be replaced, at least in part, with IL-2.

Treatment with rIL-1α 24 h prior to infection with HSV-1 provided the highest degree of protection, increasing survival to more than four times that of the control group. This 24 h period, needed to confer resistance of the newborn mice to HSV-1, coincides with the time period required for the protection of mice against *Pseudomonas aeruginosa* infection [37]. This time period allows IL-1 to activate T_h lymphocytes and macrophages, leading to an effective control of HSV-1 virus infection and spread in over 90% of the infected newborn mice.

Previous studies by Kohl et al. [16] have demonstrated that administration of recombinant human IL-2, optimally at doses of 100 U given one day prior to infection [17], provides effective immune therapy for neonatal HSV infection. Our results differ from those of Kohl [16, 17] regarding optimal dose of IL-2. We found rIL-2 at doses of 6×10^4 U/mouse to give maximal protection to neonate mice when administered one day prior to infection with HSV-1. However, differences in protection afforded by IL-2 at 6×10^3 U/mouse versus IL-2 at 6×10^4 U/mouse were not significant. An adjustment in the survival rate of mice treated with IL-2 at 6×10^4 U/mouse should be made as one set of ten mice died by day 6 post-infection and no virus was isolated from brain homogenates. However, a lethal infection of the adrenal glands cannot be ruled out as the cause of death prior to entry of the virus to the brain. If this group of ten mice is excluded from the calculations, we obtain a survival rate of 68%, instead of 54% (mouse group 7, Table 1).

Our results with M-CSF-1 revealed a slight increase in the survival rate of the newborn mice treated with the cytokine at doses of 10^3 U/mouse, but not at higher doses. Our findings suggest that M-CSF-1 stimulation of peritoneal

macrophages enhances the survival of the newborn mice and protect half of each litter against a lethal infection with HSV-1. Thus, stimulation of the immature peritoneal macrophages by M-CSF-1 has a protective value similar to that of IL-2 stimulation of immature T-cells in the newborn mice.

In this study, we showed that combinations of the recombinant cytokines IL-2 and M-CSF-1 that stimulate the immature T-cells and macrophages, respectively, provide a high degree of protection against infection with HSV-1. Survival rates greater than two times that of the control group were observed, suggesting that these two cytokines act synergistically to increase resistance of the newborn mice to HSV-1 infection. Yet, despite the marked ability of a combination of these cytokines to protect against infection, survival rates reached a maximum of only 77%, indicating that even these combination doses are not sufficient to confer full protection. Increasing the concentration of either cytokine in the combination dose appears to be ineffective, or even deleterious, (based on our results), thus the use of an additional cytokine such as IL-1α might be used to further increase survival rates. The successful protection of newborn C57BL/6 mice against HSV-1 by recombinant IL-1, as shown in the present study, might be taken to suggest that induction of the maturation of macrophages and T helper cells require the induction of intracellular molecular events in these cells which lead to the maturation of the cells and to their antiviral activity.

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